Immunological Factors Mediating the Resistance and Susceptibility to Filarial Nematodes

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That wee bit heap o' leaves an' stibble
Has cost thee mony a weary nibble!

Rabbie Burns, To a Mouse

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ABBREVIATIONS

AM	Asymptomatic Microfilaraemic
ANOVA	Analysis of Variance
bp	Base Pairs
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
Con A	Concanavalin A
CP	Chronic Pathology
cpm	Counts per minute
ddH_2O	Distillled and deionised water
DMEM	Dulbecco's Modification of Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenedaiminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
EN	Endemic Normal
FCS	Heat-inactivated foetal calf serum
FITC	Fluorescein Isothiocyanate
GLM	General Linear Modelling
HEL	Hen Egg Lysozyme
IFNγ	Interferon – gamma
IL	Interleukin
Ig	Immunoglobulin
mRNA	Messenger RNA
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PBST	PBS with 0.5% Tween

PCR	Polymerase Chain Reaction
PE	R-Phycoerythrin
PI	Post - infection
pRBC	parasitised RBC
RAG	Recombination - activation gene
RBC	Red Blood Cell
RNA	Ribonucleic Acid
SCID	Severe Combined Immunodeficiency
SDS	Sodium dodecyl sulphate
SEM	Standard Error of the Mean
TBE	Tris-borate / EDTA
TBS	Tris buffered saline
TBST	TBS with 0.5% Tween
Tris	Tris (hydroxymethyl)-aminoethane
Tween	Polyoxyethylene sorbitol monolaurate
WSP	*Wolbachia* Surface Protein

ABSTRACT

The filarial nematodes *Wuchereria bancrofti* and *Brugia malayi* are responsible for the disease lymphatic filariasis. Current estimates suggest that, in the 80 countries where this disease is endemic, around 120 million people are infected. Although not a fatal disease, the morbidity caused by lymphatic filariasis results in huge economic losses in endemic countries. Chemotherapeutic approaches to filariasis, such as targeting adult filarial nematodes using the macrofilaricidal drug diethylcarbamazine (DEC), and implementing a transmission blocking strategy using the microfilaricidal drugs albendazole and ivermectin, have been successful in reducing the prevalence of infection. However individuals in endemic areas are still susceptible to re-infection post-treatment. Therefore vaccination is likely to be required for long-term protection against filarial nematode infection.

Human filarial infection is acquired from the intermediate host, the mosquito. Most people in an endemic area are susceptible to mosquito biting yet not everyone will develop the symptoms of filarial disease. Host immune responses evoked by filarial nematodes are likely to play a role in determining the success of the establishment of filarial infection. Understanding the immunological factors that contribute to the establishment of filarial infection will help in the rational design of a vaccine that will lead to a more long-term control strategy. None of the nematodes that cause human filarial disease readily establish and develop in laboratory mice. However the rodent filarial nematode *Litomosoides sigmodontis* can complete development to patency in

BALB/c inbred mice. The studies in this thesis have utilised this rodent model to study factors that influence the establishment of filarial infection.

Filarial nematodes evoke strong type 2 host immune responses characterised by the cytokine interleukin 4 (IL4). By infecting mice that are unable to produce IL4 we have discovered that IL4 can mediate resistance to the establishment of *L. sigmodontis* infection. Infections of chimeric mice that can only produce IL4 from the innate or the acquired immune response indicate that IL4 needs to be produced from both arms of the immune system to confer protection.

We have also studied the impact of co-infecting protozoan pathogens that induce strong type 1 responses. Co-infection with the protozoan parasites *Plasmodium chabaudi* or *Leishmania major* resulted in the accelerated death of established adult *L. sigmodontis.* Immunological analysis indicates that this was correlated with a decrease in type 2- like responses against *L. sigmodontis* parasites in both cases. However we did not detect any increase in type 1-like responses against *L. sigmodontis* in either of these studies.

Finally we investigated the role of the filarial intracellular bacteria *Wolbachia.* We established that anti-*Wolbachia* immune responses do occur in human filarial infection, in individuals not treated with anti-filarial drugs. Using general linear modelling we determined that anti-*Wolbachia* surface protein (WSP) antibodies are generated mainly by the L3 stage. In support of this, analysis of life cycle stages of *L.*

sigmodontis indicate that, per gram of nematode material, the L3 stage evokes by far the greatest antibody responses against WSP. Additionally we have shown that anti-WSP immune responses can, in some circumstances, promote the establishment of primary *L. sigmodontis* infection.

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CHAPTER 1

Introduction

1:1 Lymphatic filariasis

The filarial nematodes *Wuchereria bancrofti* and *Brugia malayi* are responsible for the disease lymphatic filariasis. Current estimates suggest that in the 80 countries where this disease is endemic, around 120 million people are infected (World Health Organisation, 2000). Although not fatal, the morbidity caused by this disease results in huge economic losses in endemic countries (Haddix and Kestler 2000). Chemotherapeutic approaches to filariasis using the drug diethylcarbamazine (DEC) (Gelband 1994), albendazole (Ottesen *et al.*, 1999) and ivermectin (Brown *et al.*, 2000; Plaisier *et al.*, 2000) which mainly target the microfilarial stage and thus act to block transmission, have been successful in reducing the prevalence of infection. However individuals in endemic areas are still susceptible to re-infection post-treatment. Therefore vaccination is likely to be required for long-term protection against filarial nematode infection.

Filarial infection in humans is acquired from blood meals taken by the intermediate host, the mosquito (Fig. 1:1). Most people in an endemic area are susceptible to mosquito biting yet not everyone in an endemic area will develop the symptoms of filarial disease. Lymphatic filariasis is a spectral disease (Partono 1987) (Fig. 1:2). At

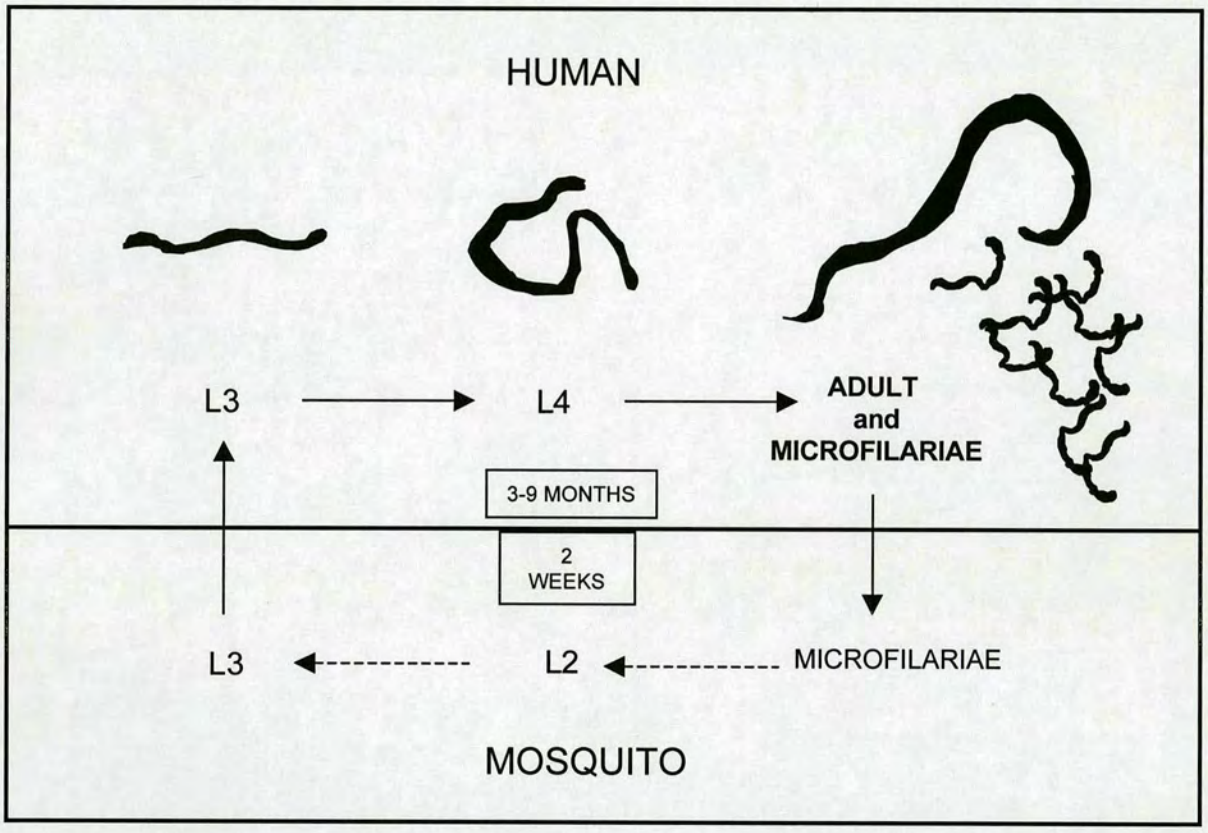
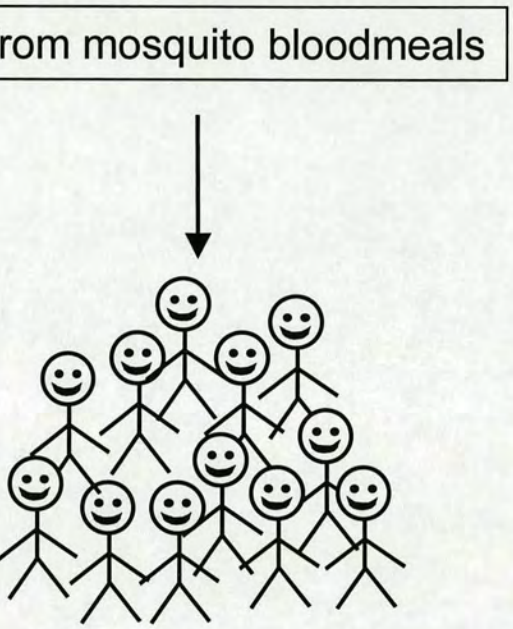


Figure 1:1 The life cycle of filarial nematodes. Infection is initiated with the L3 stage when an infected mosquito takes a blood meal. After undergoing two moults adult filarial nematodes mate and the L1 stage (microfilariae) circulate in the bloodstream. Microfilariae are ingested during a mosquito bloodmeal and undergo two moults to become L3's completing the life cycle.



ENDEMIC NORMALS	ASYMPTOMATIC MICROFILARAEMICS	CHRONIC PATHOLOGY
HEALTHY	HEALTHY	PATHOLOGY
NO SIGNS OF INFECTION	HIGH NUMBERS OF CIRCULATING BLOODSTREAM Mf	NO ACTIVE INFECTION

Figure 1:2 Lymphatic filariasis is a spectral disease. Although all individuals in an endemic area get bitten by infected mosquitos, some appear to be resistant to the establishment of filarial infection (endemic normals). Amongst susceptible individuals asymptomatic microfilariaemics appear healthy but have a high number of microfilaria circulating in their bloodstream.Others show the symptoms of chronic filarial infection, but do not appear to have an active infection.

one end of the spectrum susceptible individuals show signs of chronic pathology such as lymphadenitis, oedema, hydrocele and elephantiasis (Dreyer *et al.*, 2000). At the other end of the spectrum "resistant" endemic normals show no signs of infection, or symptoms of pathology. In the middle are the asymptomatic microfilariaemics (AM), people who appear healthy but have an established filarial infection and circulating microfilariae in their bloodstream. The ratio of individuals falling into each of these three categories varies geographically and may be linked to transmission intensity (King *et al.*, 2001).

1:2 "Resistant" and "susceptible" individuals display different anti-filarial immune responses.

The three different clinical groups display distinctly different anti-filarial immune responses (Hussain and Ottesen 1986; Ottesen 1992; Kurniawan *et al.*, 1993; Maizels 1995; Kurniawan-Atmadja *et al.*, 1998; Sartono *et al.*, 1999). One of the interesting immunological features of lymphatic filariasis is the observation of suppressed anti-filarial immune responses in those who are asymptomatic and microfilaraemic (Ottesen *et al.*, 1977). These individuals are hypothesised to be immunologically tolerant to the parasite (Maizels and Lawrence 1991). The symptoms of chronic pathology have been correlated with strong type-1 inflammatory immune responses against the adult stage of filarial nematodes (King and Nutman 1993; Addiss *et al.*, 1995; Maizels *et al.*, 1995) although it is unknown whether these observations are a cause or a consequence of adult nematode death. The immune responses mounted by

endemic normals, and how these responses mediate resistance to the establishment of filarial nematode infection, are not clearly defined (Nutman *et al.*, 1989). However some studies indicate that anti-parasite immune responses mounted in this group of "resistant" individuals, similarly to the chronic pathology groups, are type-1 inflammatory responses typified by secretion of filarial-specific IFNγ from peripheral blood mononuclear cells (PBMC) (Yazdanbakhsh *et al.*, 1993; Addiss *et al.*, 1995; Maizels *et al.*, 1995; Dimmock *et al.*, 1996; Sartono *et al.*, 1997)

Many of these studies have examined responses to adult, rather than L3, filarial antigen. Since the endemic normal group are more likely to be exposed to the L3 stage from mosquito bites, rather than adult parasites from an established filarial infection, these responses may not be reflective of why the majority of endemic normals have no active infection. Antibody responsiveness to the L3 stage does not appear to follow the same pattern of anti-adult cellular responsiveness in these three clinically defined groups of people (Day *et al.*, 1991; Bailey *et al.*, 1995). Age rather than clinical status appears to correlate most strongly with the level of anti-L3 antibodies (Day *et al.*, 1991; Helmy *et al.*, 2000).

It is unknown whether anti-L3 responses play any role in resistance to the establishment of filarial nematode infection in humans. However studies in laboratory models of filarial infection indicate that immune responses induced by the L3 stage of filarial nematodes can protect against the establishment of rodent filarial infection (Oothuman *et al.*, 1979; Yates and Higashi 1986; Lucius *et al.*, 1991: Weil *et al.*,

1992; Le Goff *et al.*, 2000a). The protective immune responses associated with L3's in these laboratory vaccination models have been associated with high interleukin 5 (IL5) production (Bancroft *et al.*, 1993; Le Goff *et al.*, 2000a; Le Goff *et al.*, 2000b; Martin *et al.*, 2000a), a cytokine that has been associated with accumulation of neutrophils around adult filarial nematodes (Al-Qaoud *et al.*, 2000).

1.3 *Litomosoides sigmodontis* infection of laboratory mice as a model filarial infection

Understanding the immune responses evoked by filarial nematodes that play a role in determining the success of the establishment of filarial infection will help in the rational design of a vaccine. It is not possible to investigate the establishment of primary filarial nematode infection in humans for ethical reasons. Therefore research investigating immune responses evoked during the establishment of filarial nematodes in primary infection depends on the use of animal models.

1:3:1 Infectivity of human disease-causing filarial nematodes in laboratory animals.

Utilisation of human disease-causing filarial nematodes in laboratory animals has been hampered because neither *W. bancrofti*, responsible for around 90% of lymphatic filarial infection (World Health Organisation, 2000), nor *Onchocerca volvulus*, the main causative agent of Onchocerciasis (World Health Organisation 2000), survive in any of the laboratory rodents examined so far (Suswillo *et al.*, 1977; Kozek *et al.*,

1982). *Mansonella ozzardi*, a filarial parasite that is very similar to *O. volvulus* and endemic in Latin America also does not survive in jirds (Suswillo *et al.*, 1977). *Loa loa* is a human filarial parasite that is endemic in west and central Africa and causes pathological sequelae in humans with similarities to both lymphatic filarial nematodes (formation of hydroceles) (Akue and Devaney 2002) and *Onchocerca volvulus* (ocular inflammation) (Pinder *et al.*, 1988). Simlarly to the other human disease-causing filarial parasites this parasite does not survive beyond a week in jirds or mice (Suswillo *et al.*, 1977; Wanji *et al.*, 2002).

Brugia malayi, the filarial nematode species causing lymphatic filariasis in South East Asia, can be maintained in laboratory jirds *(Meriones unguiculatus)*. Unfortunately a lack of jird-specific immunological reagents has made it time-consuming and very difficult to examine immune responses in this animal model. Alternatively the survival of human infective filarial nematodes has been studied in primates, a close cousin of *Homo sapiens*. Almost all human filarial nematodes will establish and survive in primates (*W. bancrofti*: Rao *et al.*, 1980; Campbell *et al.*, 1987: *O. volvulus*: Kozek *et al.*, 1982; Greene 1987: *B. malayi*: Wong *et al.*, 1977: *M. ozzardi*: Orihel *et al.*, 1981; Orihel *et al.*, 1993: *Loa loa*: Orihel and Moore 1975; Orihel and Eberhard 1985: General review: Schacher *et al.*, 1973; Kumar *et al.*, 1991). However the logistics of undertaking experiments in primates make this option expensive and conclusions often need to be drawn from a small number of animals in each experimental group. A further difficulty to using this system is the ethical constraints in the use of primates in medical research.

Due to the difficulties cited above, research into anti-filarial immune responses using animal models has centred around immune responses evoked by individual stages of filarial nematodes. Laboratory mice, for which there is an abundance of immunological reagents to study immune responses, have successfully been used to investigate stage specific immune responses induced by the adult and microfilarial stages of *B. malayi* (Lawrence *et al.*, 1994; Allen and MacDonald 1998). Adult stages are normally implanted in the peritoneum of mice where they can live in excess of 4 weeks (Lawrence *et al.* 1994; Lawrence *et al.*, 1995) and microfilariae can be injected intravenously and will circulate for up to 9 weeks until they are cleared by the immune system (Fanning *et al.*, 1983). Nevertheless it is not possible to study the establishment of filarial nematodes using this parasite system as L3 stages do not survive in inmunocompetent mice in any of the strains tested.

1:3:2 The use of animal filarial nematodes to study anti-filarial immune responses.

To study and manipulate immune responses that may be involved in the establishment of filarial nematodes, some studies have utilised animal filarial nematodes that can establish and complete their life cycle in their natural hosts. *Onchocerca ochengi* is a bovine onchocercid that can establish and complete its life cycle in cows. This parasite has been successfully used as a model for human onchocerciasis (Trees *et al.*, 2000). Alternatively filarial nematodes of dogs and cats (primarily *Dirofilaria immitis* and *Brugia pahangi)* (Donahoe 1975; Denham and Suswillo 1980; Abramowsky *et al.*, 1981; Rawlings 1982; Weil *et al.*, 1982) could be used in their natural host to study

immune responses involved in establishment. However, as for primate research, the main problems with research in dogs and cats are the logistical and ethical issues that prevent the wide use of these animals, as well as the immunological reagents required.

Acanthocheilonema vitea is a natural parasite of rodents and will complete its life cycle in jirds (Lucius *et al.*, 1995). However, as with *B. malayi*, the studies using this model are also limited by the paucity of anti-jird immuno-reagents although this model has successfully been used in some studies of anti-L3 responses (Lucius *et al.*, 1991; Bleiss *et al.*, 2002).

1:3:3 The use of the *Litomosoides sigmodontis* murine model in examining immune responses to establishing filarial nematodes.

The studies in this thesis have made use of the *Litomosoides sigmodontis* rodent filarial nematode to study factors that influence the establishment of filarial infection. This main advantage of this model over the other possible models of filariasis is that it can complete its life cycle in BALB/c laboratory mice (Petit *et al.*, 1992; Bain *et al.*, 1994: Hoffman *et al.*, 2000). Therefore this model allows the study of immune dynamics across the entire course of infection. The murine immune system is by far one of the most well-characterised mammalian immune systems and there is an abundance of reagents, in addition to a range of mice genetically deficient in individual immune components, that allow analysis of immune responses. Although BALB/c mice are susceptible hosts for to the establishment of infection with *L. sigmodontis*, most other mouse strains are resistant (Petit *et al.*, 1992; Marechal *et al.*,

1996). Therefore the immune responses evoked by *L. sigmodontis* in resistant and susceptible hosts can also be compared.

L. sigmodontis is a parasite that is transmitted by the mite species *Ornithonyssus bacoti* and found in wild cotton rats (*Sigmodon hispidus*). Infection can be initiated with approximately 25 L3 removed from mites and injected into mice subcutaneously. Within 10 days the L3 stages can already be found in the thoracic cavity. The parasites moult a further two times in the thoracic cavity to become adult parasites (L5) at which point they mate to produce microfilariae (L1). Unlike the microfilariae produced from *B. malayi* adults implanted in mice (Lawrence *et al.*, 1994), the microfilariae of *L. sigmodontis* circulate in the blood stream. Microfilariae are produced from approximately day 50 post-infection and are taken up my mites where they moult twice over a two week period to become L3's completing the life cycle.

L. sigmodontis infection of mice offers many opportunities to examine immune responses in filarial infection that are difficult or impossible to study in other filarial models. However some differences must exist between the different species of filarial nematodes to confer host specificity. The genetic variation between different species of filarial nematodes creates the potential to generate subtly different immune responses. However *L. sigmodontis* has been shown to have significant immunological cross-reactivity to *W. bancrofti*, *O. volvulus*, *B. malayi* and *Loa loa* (Harnett *et al.*, 1989; Marechal *et al.*, 1994). Additionally it has been demonstrated that *L. sigmodontis* L3's express some of the same genes as the L3 stage of *B. malayi* and *O.*

volvulus (Allen *et al.*, 2000). Migration to the thoracic cavity by *L. sigmodontis* in rodents is different from migration and residence in the lymphatic vessels (*W. bancrofti, B. malayi*) or the skin (*O. volvulus*) in human infection. Different sites in the body can be predisposed to inducing different types of immune responses (Constant *et al.*, 2000) and therefore migration through different tissues by different species of nematode may evoke different immune responses. Nevertheless one study has determined that, although the L3 stages of human filarial nematodes do not survive very long in rodents, they appear to have a similar pattern of migration to *L. sigmodontis* L3 in these animals (Bain *et al.*, 1994). Overall it can be said that *L. sigmodontis* has sufficient genetic similarity and immunological cross-reactivity to the human filarial nematodes to provide useful information on the immune responses evoked in primary filarial nematode infection.

1:3:4 Immune responses evoked by *L. sigmodontis* in susceptible and resistant mice.

The immune responses evoked by *L. sigmodontis* in susceptible BALB/c mice are characterised by the production of a number of type 2-associated cytokines such as interleukin 4 (IL4), IL5, IL6 and IL10 (Marechal *et al.*, 1997; Le Goff *et al.*, 2000a) in addition to the type 1 cytokine interferon-γ (IFNγ) (Le Goff *et al.*, 2000a). Studies of the cytokines induced by *L. sigmodontis*, and how the mediate resistance and susceptibility to infection, are still in their infancy. However Marechal *et al.*, (1997) observed that resistance in B10.D2 was associated with the early appearance of circulating anti-*L. sigmodontis* antibodies when compared with BALB/c mice. This

indicates that early immune responses may be an important determinant in nematode survival in these laboratory mice.

1:4 Immunological factors investigated

1:4:1 Interleukin 4 (Chapters 3 & 4)

1:4:1:1 IL4 responses in filarial infection

It has long been recognised that filarial nematodes evoke strong T helper (Th) 2 – like immune responses in both human and murine infection (for review see Ottesen, 1992; Lawrence and Devaney, 2001). Such a response is typified, and is also driven, by the production of IL4. Similar levels of IL4 are secreted from PBMC of all three human clinical groups (reviewed by Maizels *et al.*, 1995) and therefore it is currently unknown whether the IL4 response evoked by filarial nematodes plays any role in resistance or susceptibility to filarial nematode infection.

The IL4 response in filarial nematode infection can occur within hours of infection with L3 stage (Osborne and Devaney 1998; Balmer and Devaney 2002) and is a feature of *L. sigmodontis* infection (Marechal *et al.*, 1997; Le Goff *et al.*, 2000a). Therefore we have been able to use the *L. sigmodontis* model to investigate the impact of this response on the development of the L3 stage to adults and the establishment of primary filarial nematode infection. The resistance of laboratory mice against the gut nematodes *Trichuris muris* (Else *et al.*, 1994; Bancroft *et al.*, 1998) and *Heligosomoides polygyrus* (Urban *et al.*, 1991) has been shown to be

dependent on IL4, although the exact mechanisms by which this occurs are still under investigation (for review see Finkelman *et al.*, 1997). We have assessed the necessity of IL4 production for resistance to filarial nematode establishment using *L. sigmodontis* infections of C57BL/6 mice that are deficient in IL4.

1:4:1:2 Immunological sources of IL4

IL4 can come from a variety of sources in the immune system including both cells of the innate and adaptive immune system. Polymorphonuclear granulocytes such as mast cells (Weiss and Brown 2001), basophils (Brunner *et al.*, 1993; Arock *et al.*, 1993) and neutrophils (Brandt *et al.*, 2000) are all involved in the innate immune response and are capable of producing IL4 that can potentially direct an immediate Th 2-type immune response. T cells are one of the main populations of cells producing IL4. NKT cells are a population of cells positive for CD4 and NK 1.1 that have been shown to produce large amounts of IL4 when stimulated by antigen through their limited repertoire of T cell receptor's (TCR's) (Yoshimoto *et al.*, 1995; Chen and Paul 1997). γδ T cells are a small population of circulating lymphocytes that populate mainly the epithelial tissues such as the skin, intestine and the reproductive tract. This population of cells can produce IL4 *in vivo* (Ferrick *et al.*, 1995). Furthermore when CD4+ and CD8 + cells are stimulated with antigen in the presence of IL4, they begin to produce IL4 forming a positive feedback loop that maintains the type 2 phenotype of the immune response. As IL4 can come from both the innate and the adaptive arms of the immune system, and IL4 is strongly induced by *L. sigmodontis* infection, we have analysed the relative contributions of IL4

production from the innate and adaptive arms of the immune response of the establishment of *L. sigmodontis*.

1:4:1:3 Actions of IL4 in the immune response

IL4 is a cytokine that induces upregulation of MHC Class II molecules (Noelle *et al.*, 1984) as well as the isotype switching of human B cells to produce IgG4 and IgE (Gascan *et al.*, 1991). Both filarial-specific IgE and polyclonal IgE can be found in the bloodstream of all the clinical groups of infected individuals (Kurniawan *et al.*, 1993; Yazdanbakhsh *et al.*, 1993; Bal and Das 1999; Nicolas *et al.*, 1999; Terhell *et al.*, 2002; Lobos *et al.*, 2003) This isotype can mediate the degranulation of granulocytes such as mast cells and basophils through cross-linking of the FcεRI (Marone *et al.*,1999), an action that could potentially harm both invading and established filarial nematodes.

1:4:1:4 Production of anti-*L. sigmodontis* cytokines in the absence of IL4.

IL4 has the potential to down-regulate Th1 type 1 inflammatory responses (Abbas *et al.*, 1996; Mosmann and Sad 1996; O'Garra 1998) (see 1:4:2:3) that have been hypothesised to mediate filarial nematode development (Ravindran 2001).

To assess the immune response evoked by *L. sigmodontis* in the absence of IL4, we have measured the type 1 signature cytokine IFNγ as well as the down-regulatory cytokine IL10 in response to *L. sigmodontis* at day 60 post-infection.

1:4:1:4:1 IFNγ

NK cells are an important part of the innate immune response and are recruited to the site of infection almost immediately after pathogen invasion (Bancroft 1993). NK cells are an important source of IFNγ in innate immune responses. Similarly to IL4, NK 1.1 CD4+ T cells (Chen and Paul 1997), as well as γδ T cells (Yin *et al.*, 2000), have been shown to secrete large amounts of IFNγ upon activation. Mϕ and DC's are capable of producing IFNγ although this is more pronounced in the presence of IL12 and IL18 (Stober *et al.*, 2001, Munder *et al.* 1998; Fukao *et al.*, 2000). CD4+ Th1 cells are the main producers of IFNγ in the adaptive immune system, and produce IFNγ when stimulated with antigen in the presence of IL12 and IL18 (Micallef *et al.*, 1996; Barbulescu *et al.*, 1998).

IFNγ can up-regulate the expression of MHC I and MHC II molecules on macrophages and other cell types, as well as induce respiratory burst via induction of macrophage genes leading to the production of the anti-microbial products nitric oxide and superoxide (Boehm *et al.*, 1997). Macrophages also upregulate expression of the FcγR1 in response to IFNγ (Perussia *et al.*, 1983; Guyre *et al.*, 1983) which is a surface receptor that binds with high affinity to IgG2a antibodies, the isotype produced by murine B cells in response to IFNγ (Snapper and Paul 1987a; Collins and Dunnick 1993). Thus IFNγ coordinates the regulation of Fc receptors and antibody ligand facilitating increased phagocytosis of pathogens opsonised by IgG2a. IFNγ can synergise with other inflammatory cytokines such as TNFα, IL1 and IL6 to

activate the acute phase response which controls fever (Morimoto *et al.*, 1987), and also trigger the transcription of complement genes in the liver (Volanakis 1995).

1:4:1:4:2 IL10

IL10 is made by a wide variety of cells including T regulatory cells, Th2 cells and Th1 cells (at least in mouse), as well as dendritic cells (DC's) expressing CD8α (Grohmann *et al.*, 1999) and DC's found in the Peyers patches (Iwasaki and Kelsall 1999) and the liver (Khanna *et al.*, 2000). IL10 is also secreted by activated B cells (Burdin *et al.*, 1995) and prolongs their survival (Ito and Hirohata 1995), possibly due to up-regulation of the anti-apoptotic marker bcl-2 (Levy and Brouet 1994). The antibody isotype switching of human naïve B cells activated by CD40 to IgG1 and IgG3 (Malisan *et al.*, 1996) and of murine B cells stimulated with LPS to IgG3 (Shparago *et al.*, 1996) is also mediated by IL10.

One of the main mechanisms that IL10 from the adaptive system is able to down-regulate the immune response is by reducing expression of cytokines, soluble mediators and cell surface molecules via antigen presenting cells (APC's) such as dendritic cells and macrophages (for review see Moore *et al.*, 2001). In macrophages IL10 can down-regulate the production of the inflammatory cytokines IL1, IL6 and TNFα (Fiorentino *et al.*, 1991). Furthermore the surface expression of co-stimulatory molecules such as CD80/CD86 (B7.1 /B7.2) on macrophages is also down-regulated in response to IL10 (Ding *et al.*, 1993) reducing the ability of these macrophages to deliver co-stimulatory signals to T cells. The absence of this second co-stimulatory

signal during T cell activation has been linked to the induction of tolerance in the T cells (Sebille *et al.*, 2001). Indeed studies examining IL10 treated DC's indicate that they induce tolerance, rather than activate, the T cells they come into contact with (Groux *et al.*, 1996; Steinbrink *et al.*, 1997). IL10 also has profound effects on neutrophils which are one of the most abundant peripheral cell types circulating in the blood. Similarly to macrophages IL10 reduces the amount of proinflammatory cytokines released by neutrophils (Cassatella *et al.*, 1993). IL10 is also able to reduce the secretion of chemokines by neutrophils (Kasma *et al.*, 1994). Chemokines function to attract cells to the site of invasion amplifying the immune response. By reducing chemokine secretion by neutrophils, IL10 again acts to down-regulate the immune response.

1:4:1:5 Cellular receptors mediating the actions of IL4, IFNγ and IL10.

Cytokines such as IL4, IFNγ and IL10 mediate their effects on target cells via receptors present on the cell surface of target cells. Triggering of cell receptors via cytokine binding leads to receptor multimerization and activation of intracellular signalling pathways which, in turn, activate transcription factors in the nucleus leading to the transcription of specific immune response genes. Many cytokine receptors activate transcription factors via the Janus kinase / signal transducer and activator of transcription (Jak/STAT) signalling pathway. The receptors are able to target specific cytokine gene transcription via tyrosine phosphorylation of specific Jak and STAT molecules as detailed below (also Figure 1:3).

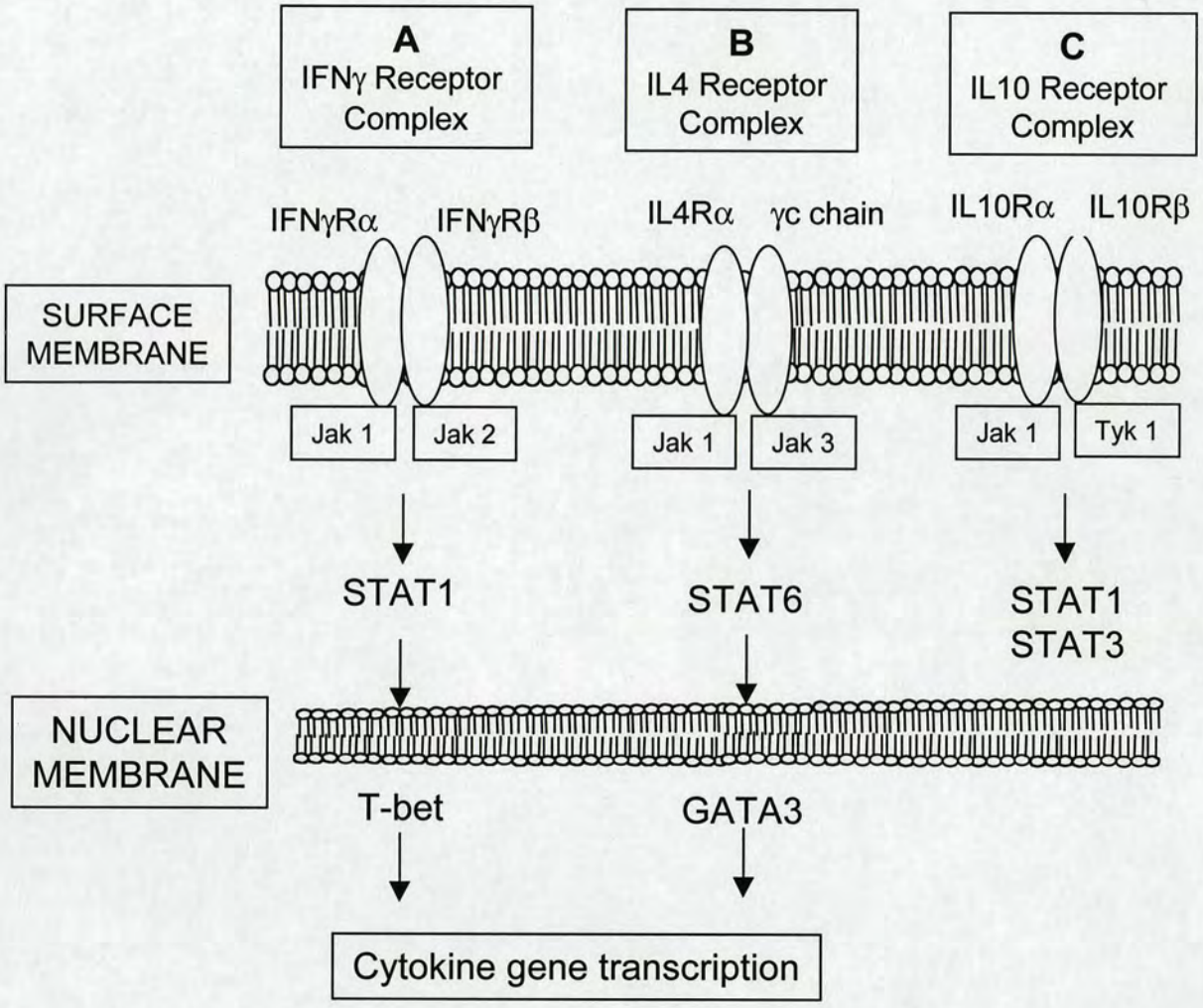


Figure 1:3 Cytokines signal through specific receptors on the surface of target cells. The IFNγR complex (A) is associated with Jak 1 and Jak 2 which phosphorylate STAT1 leading to the activation of T-bet. The IL4 complex (B) is associated with Jak 1 and Jak3 which phosphorylate STAT6 and activate GATA3. The IL10 complex (C) is associated with Jak1 and Tyk1 which phosphorylate STAT1 and STAT3.

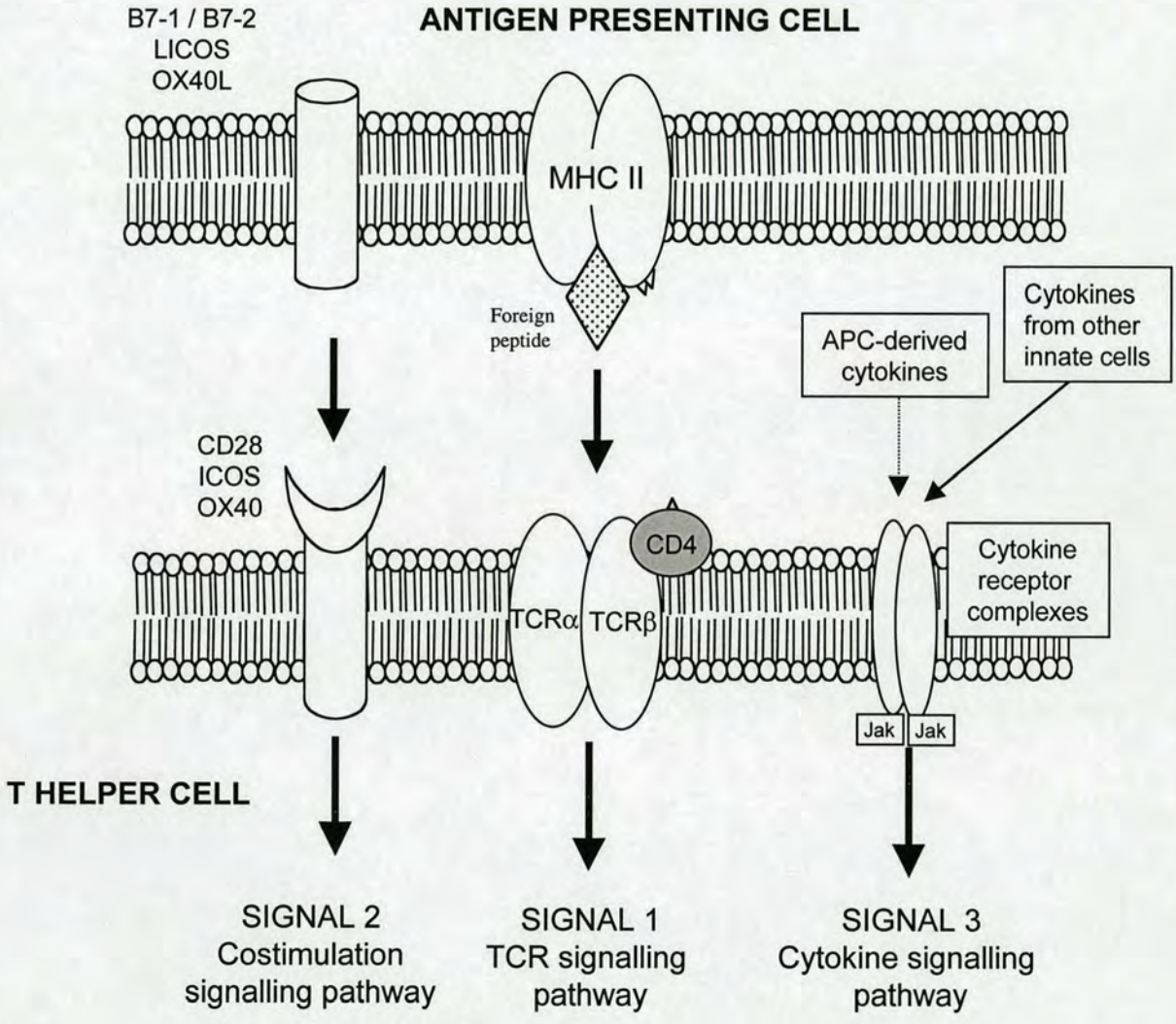


Figure 1:4 The three signals involved in the initiation and direction of T helper cells in adaptive immune responses. The T cell receptor (TCR) is composed of two chains and recognises a specific peptide presented by the antigen presenting cell in the context of MHC class II molecules. CD4 is a co-receptor molecule which binds to MHC II to help stabilise the interaction. Triggering of the TCR complex activates intracellular signalling pathways associated with the TCR (signal 1). Simultaneously the antigen presenting cell up-regulates co-stimulatory ligands that can bind to co-stimulatory molecules on the surface of the T cell. This action activates intracellular pathways associated with these co-stimulatory molecules providing signal 2. Thirdly cytokines secreted from the APC as well as other innate system cells such as polymorphonuclear granulocytes triggers cytokine receptors and their associated signalling pathways polarising the T cell towards Th1 or Th2 (signal 3).

The IL4 receptor α (IL4Rα) is a member of the haematopoietin receptor superfamily that requires heterodimerization with the common-γ chain (γc chain) in order to signal. IL13 also signals through the IL4Rα but requires IL13Rα rather than the γc chain to form a functional receptor complex. IL4R engagement results in tyrosine phosphorylation of Jak 1, which is associated with the IL4Rα chain, and Jak 3 which associates with the γc chain (Miyazaki *et al.*, 1994; Russell *et al.*, 1994). Phosphorylation of STAT6 by Jak1 and Jak 3 leads to the expression of IL4 responsive genes (Zhu *et al.*, 2001). The transcription factor GATA3, a major controller of Th2 differentiation, is also activated by activation of STAT6 (Zheng and Flavell 1997; Kurata *et al.*, 1999).

The receptor for IFNγ is composed of two receptor subunits, IFNγRα and IFNγRβ. These receptor chains are associated with Jak1 and Jak 2 respectively (Kaplan *et al.*, 1996; Sakatsume *et al.*, 1995). Phosphorylation of Jak1 andJak2 by IFNγ engagement of the IFNγR complex results in the phosphorylation of STAT1 and the activation of T-bet (T-box expressed in T cells), a transcription factor that can activate the expression of IFNγ and is associated with differentiation of T cells into Th1 type cells (Szabo *et al.*, 2000, Mullen *et al.*, 2001). Therefore the triggering of the IFNγR complex on T cells can act in an autocrine manner to amplify IFNγ responses in a positive feedback loop.

IL10 exerts its effects by binding to the IL10R complex, which similarly to the IFNγ receptor complex consists of 2 chains, IL10Rα and IL10Rβ both of which are members of the interferon receptor (IFNR) family. Interaction between IL10 and the IL10 receptor complex induces phosphorylation of Jak1 and Tyk1 associated with the IL10R complex (Finbloom and Winestock 1995). This is followed by the subsequent tyrosine phosphorylation and activation of the transcription factors STAT3, STAT1 and, in non-macrophage cells, STAT5 (Finbloom and Winestock 1995; Wehinger *et al.*, 1996).

1:4:2 Immune responses evoked by co-infecting pathogens (Chapters 5 & 6)

There is geographical overlap between the endemicity of filarial nematodes and protozoan parasites such as *Plasmodium*, the causative agent of malaria. Protozoan parasites generally induce strong type 1 responses typified by the inflammatory cytokine IFNγ. Anti-filarial type 1 inflammatory responses have been shown to be down-regulated in individuals with active filarial nematode infection (Maizels *et al.*, 1995; Sartono *et al.*, 1997). We hypothesised that immune responses generated by co-infecting protozoan pathogens may interact with immune responses to established filarial nematode infection. Such immune responses may act as an immunological factor that could influence resistance and susceptibility to filarial nematode infection. We have examined the effect of immune responses generated against the protozoan parasites *Plasmodium chabaudi* and *Leishmania major* on the outcome of *L. sigmodontis* infection and anti-filarial immune responses.

1:4:2:1 *Plasmodium chabaudi*

Plasmodium chabaudi is a natural parasite of thicket rats (*Thamnomys rutilans*) which infects laboratory mice. The parasite multiplies asexually in circulating host red blood cells every 24 hours, causing anaemia, weight loss and cachexia (see Cox, 1988). Some of these symptoms are due to direct parasite factors such as red blood cell destruction at shizogeny, however components of the inflammatory immune response evoked by *P. chabaudi* can also directly aggravate symptoms of malaria for example by causing anaemia due to diserythropoiesis (Mohan and Stevenson 1998).

P. chabaudi parasites can directly activate dendritic cells to produce the pro-inflammatory cytokines IL12, TNF and IL6 (Seixas *et al.*, 2001). Correspondingly TNFα and the type 1 signature cytokine IFNγ can be measured in the serum of infected mice within the first week post-infection (Stevenson and Tam 1993; Stevenson *et al.*, 1995; Cross and Langhorne 1998) and from CD4+ T helper cells in the spleen (Taylor Robinson *et al.*, 1994). After the level of parasitaemia peaks (around day 14 post-infection), the initial type 1 immune response to *P. chabaudi* becomes more mixed with the appearance of parasite specific T cells secreting IL4 (Langhorne and Simon 1989; Taylor-Robinson 1994).

1:4:2:2 *Leishmania major*

Leishmania major is a parasite causing cutaneous leishmaniasis in humans. Most cases of cutaneous leishmaniasis occur in Iran, Afghanistan, Syria, Saudi Arabia, Brazil and Peru (World Health Organisation 2000). *L. major* can infect laboratory

mice causing footpad lesions when injected sub-cutaneously into the footpad. In the case of C57BL/6 mice this lesion resolves around 10-12 weeks post-infection (Matthews *et al.*, 2000). However BALB/c mice are unable to control infection and the infection becomes fatal when parasites disseminate from the lesion (Nasseri and Modabber 1979). Thus infection of these laboratory strains of mouse with *L. major* follows the same resistant / susceptibility phenotype as infection with *L. sigmodontis*.

Early studies into the immune responses of BALB/c mice to *L. major* infection indicated that susceptibility was correlated with an IL4 response evoked by the parasites (Sadick *et al.*, 1990; Chatelain *et al.*, 1992; Kopf *et al.*, 1996). However *L. major* has been shown to induce IL4 in resistant strains of mice (Morris *et al.*, 1992; Reiner *et al.*, 1994; Heinzel *et al.*, 1995: Scott *et al.*, 1996). The difference between resistance and susceptibility is now thought be due to the ability of the mouse to redirect this type 2 response, characterised by IL4 production, into a type 1 response, characterised by IFNγ production, via the induction of IL12 (Sypek *et al.*, 1993; Park *et al.*, 2000; Hondowicz *et al.*, 2000). Evidence that type 1 responses are involved in resistance to *L. major* comes from infections of resistant mice deficient in IL12 (Mattner *et al.*, 1996; Park *et al.*, 2000), IFNγ (Wang *et al.*, 1994a and 1994b) and the type 1 transcription factors T bet and STAT4 (Szabo *et al.*, 2002; Stamm *et al.*, 1999) (see 1:4:2:7:1) which become susceptible to infection and are unable to clear their lesions.

1:4:2:3 The initiation of immune responses.

The immune response begins when pathogens are recognised by receptors on the surface of APC's recognising specific motifs, or pathogen associated molecular patterns (PAMP's). The most widely characterised innate recognition receptors and the Toll family of receptors (TLR). There are at least 10 different TLR's in humans (Rock *et al.*, 1998; Takeuchi *et al.*, 1999; CHuang and Ulevitch 2000; Chuang and Ulevitch 2001) which recognise a range of pathogen molecules including lipoproteins (TLR2, Lien *et al.*, 1999; Hirschfeld *et al.*, 1999), LPS (TLR4, Poltorak *et al.*, 1998; Hoshino *et al.*, 1999) and CpG DNA (TLR9, Hemmi *et al.*, 2000). Activation of TLR's plays an important role in the maturation of dendritic cells leading to the production of cytokines, expression of co-stimulatory ligands such as CD80/CD86 and the production of pro-inflammatory cytokines such as IL12 (Akira *et al.*, 2000).

1:4:2:4 Activation of T helper cells and the adaptive immune response

Once DC's have matured they lose their capacity for phagocytosis and migrate to the draining lymph nodes where they present pathogen-derived peptides to T cells in the context of major histocompatibility molecules (MHC) molecules on the cell surface (Banchereau and Steinman 1998; Reis e Sousa 2001). There are two types of MHC molecules. MHC class I obtains peptides from antigens processed by the proteosome which come mainly from intracellular pathogens. Peptides presented on MHC I molecules activate T cells that are CD8 +ve, (cytotoxic T cells). On the other hand peptides presented on MHC class II molecules normally exogenous, are acquired

through endocytosis of antigen presenting cells (APC) and activate T cells that are CD4 +ve (T helper cells). Specificity between MHC class of molecules and the type of T cell activated is offered partly by the ability of CD8 co-receptor to bind to MHC I and CD4 co-receptor to bind to MHC II (Janeway 1992). Pathogen derived peptides in combination with MHC molecules are recognised by peptide-specific TCR's providing an activation signal for the T cell.

Naïve T cells circulate throughout the body via the bloodstream and the secondary lymphoid organs. It is in the lymphoid organs that naïve T cells encounter APC's with pathogen-specific peptides displayed on their surface in combination with MHC molecules. The specificity of the TCR determines the specificity of each T çell. However T cells that bear the appropriate TCR for the presented peptides will not become activated unless they also receive a second "co-stimulatory" signal (Fig. 1:4). There are a variety of co-stimulatory molecules on the surface of T cells that will engage with the appropriate ligands found on the surface of the APC. By far the most widely characterised co-stimulatory interaction is that between CD28 and CTLA4 on the T cell and the APC expressed CD80/CD86. Other co-stimulatory molecules include ICOS / LICOS and OX40 /OX40L. After T cells have been primed through activation of signalling pathways emanating from both the TCR and the co-stimulatory molecule(s), clonal expansion of T cells occurs whereby the T cells divide generating a pool T cells with specificity to the invading pathogen.

1:4:2:5 Factors influencing the development of type 1 and type 2 responses.

Upon activation of T cells by APC's presenting peptide specific for the TCR, and co-stimulation via engagement of co-stimulatory receptors with their ligands on the APC, the T cells can secrete cytokines and other molecules that will shape the immune response to activate effector mechanisms. Different effector mechanisms will be activated by type 1 and type 2 cytokines and there are many factors that may influence whether T cells becomes Th1 or Th2 to activate the effector mechanisms associated with these types response.

1:4:2:5:1 The cytokine milieu

A major factor in the development of Th1 or Th2 cells is the cytokine milieu present at the time of activation (sometimes referred to as signal 3, Fig 1:4). Type 1 immune responses are normally generated when pathogens trigger the production of IL12 from DC's via triggering the TLR's, and IFNγ from innate sources such as NK cells. Naïve T cells do not constitutively express the IL12 receptor β chain (Wenner *et al.*, 1996; Rogge *et al.*, 1997) but this receptor is upregulated via signalling pathways emanating from the engagement and activation of the TCR (Szabo *et al.*, 2003). Triggering of the expressed IL12R complex which, like the IFNγR complex, is coupled to a Jak-STAT signalling pathway, results in the phosphorylation of STAT4. The activation of STAT4 via IL12R signalling is not essential for initial IFNγ expression (Kaplan *et al.*, 1998; Yang *et al.*, 1999; Oxenius *et al.*, 1999) but may be required to boost the level of IFNγ produced in the immune response by individual cells (Mullen *et al.*, 2001), and to maintain type 1 responsiveness (Magram *et al.*, 1996; Park *et al.*, 2000). Indeed

it has previously been shown *in vitro* that IFNγ alone cannot sustain Th1 development in the absence of IL12 (Wenner *et al.*, 1996). IFNγR's are expressed on naïve T cells (Farrar and Schreiber 1993) and engagement of the IFNγR activates the transcription factors STAT1 (Darnell *et al.*, 1994) and subsequently T box expressed in T cells (T-bet) (Lighvani *et al.*, 2001; Afkarian *et al.*, 2002).

On the other hand the presence of IL4 upon T cell priming activates the IL4R complexes endogenously expressed on naïve T cells, activating STAT6 and transcription of STAT6 regulated genes. IL4 induces an autocrine loop whereby IL4R engagement and activation of STAT6 will promote further secretion of IL4 because STAT6 binds to the IL4 promoter (Lederer *et al.*, 1996).

Cytokines are pleiotropic providing growth signals for T cells in addition to polarisation. It is unclear whether cytokines promote the development of type 1and type 2 responses purely by polarising the T cells during priming, or whether they also select for the outgrowth of the cells they polarise (reviewed by Reiner and Seder 1999; Coffman and Reiner 1999). Indeed naive T cells express gene transcripts for both IL4 and IFNγ within 1 hour of stimulation, regardless of type 1 or type 2 conditions (Grogan *et al.*, 2001). Further it has been shown that a small percentage of T cells activated under type 2 conditions still express IFNγ, rather than IL4, and conversely that there are IL4-producing cells that persist despite activation under type 1 conditions (Bucy *et al.*, 1995; Kelso *et al.*, 1995; Kelso *et al.*, 1999). This data indicates that polarising conditions are not 100% effective in polarising all T cells.

Furthermore the cytokine milieu is often less polarised *in vivo* than *in vitro* experiments leading to an even more heterogeneous population of cells in many situations.

1:4:2:5:2 The kind of signals received from APC's

The type of activation signals can also affect which type of CD4+ T helper cell will develop. Firstly there have been several studies implicating the existence of different types of DC's (DC1 and DC2) that will activate T cells differently resulting in Th1 or Th2 cells respectively (Pulendran *et al.*, 1999; Rissoan *et al.*, 1999; Maldonado-Lopez *et al.*, 1999; Kanna *et al.*, 2000; Moser and Murphy 2000; Liu 2001; Blom *et al.*, 2002). However there is also evidence that DC's are flexible and direct type 1 or type 2 responses depending on the stimulus of innate immune receptors (or PAMP's) (MacDonald *et al.*, 2001; Edwards *et al.*, 2002; Manickasingham *et al.*, 2003). Further the location of the dendritic cell when it is activated in the body can also influence the response of a dendritic cell to the same TLR (Ito *et al.*, 2002). Although all TLR's characterised thus far appear to direct type 1 immune responses, there may also be similar innate recognition receptors that direct Th2- type responses by recognition of "Th2 associated" PAMP's.

The second or co-stimulatory signal is another potential way that differential activation of DC's may result in differential outcome of T helper cell type. For example it has been reported that the stimulation of CD28 by B7-1 can induce T cells to produce IFNγ whereas B7-2 can preferentially induce T cells to secrete IL4

(Freeman *et al.*, 1995). Similarly co-stimulation via stimulation of OX40 by APC - expressed OX40L (Flynn *et al.*, 1998; Ohshima *et al.*, 1998; Tanaka *et al.*, 2000) and stimulation of ICOS via LICOS (or B7RP-1) on APC's (Brodie *et al.*, 2000; Coyle *et al.*, 2000) may favour the development of Th2 cells. However, none of these molecules are sufficient to induce terminal T helper cell differentiation in the absence of differentiation-inducing cytokines

1:4:2:5:3 The strength of the signal received from APC's

The levels of stimulation a T cell can receives can involve a variety of aspects of triggering of the TCR. The duration of the signal through the TCR can affect the polarization of T cells, with longer interactions leading to type 2 conditions (Iezzi *et al.*, 1999). This can be affected by the affinity of the peptide presented to the T cell, with high affinity peptides favouring IFNγ secretion and lower affinity peptides favouring IL4 secretion (Tao *et al.*, 1997; Rogers and Croft 1999). The strength of the signal received by the TCR can also be influenced by the density of the TCR and MHC-peptide interactions occurring (Badou *et al.*, 2001). In this study a higher density of interactions lead to IFNγ secreting cells and whilst a lower density of interactions lead to IL4 production. This has also been demonstrated with the addition of different concentrations of antigen to cell cultures whereby low doses favoured the development of IL4 secreting cells and higher doses favoured the development of IFNγ secreting cells (Hosken *et al.*, 1995). The effects of the strength of TCR signal on differentiation of T helper cell subsets has been reported to be mediated by

interactions of CD40L on the T cell with CD40 on the antigen presenting cell (Ruedl *et al.*, 2000).

1:4:2:6 Cross-regulation between polarised immune responses

In these co-infection experiments we are examining the effect of inducing a type 1 response (against *P. chabaudi* or *L. major*) on an established type 2 response (against *L. sigmodontis*). We have measured IL4 as a marker of the type 2 response and IFNγ as a marker of the type 1 response. It is possible that inducing a type 1 response will not have any affect on an established type 2 response. In this scenario it is envisaged that the immune response would become more heterogeneous by IFNγ instructing new naïve cells that are continuously being primed to become type 1 in addition to the Th2 cells already present in the established type 2 response. However since APC-derived cytokines appear to have a greater effect on the development of a Th1 phenotype (Maldonado-Lopez *et al.*, 2001) than bystander cytokine effects, incoming cytokines may have to exert this effect on via the APC rather than on the T helper cell. A heterogenous immune response could also occur under the stochastic / selection hypothesis (Reiner and Seder 1999; Coffman and Reiner 1999) whereby cytokines promote the outgrowth of polarised T cells from a heterogenous population. The developing type 1 response would again result in a mixed population of type 1 and type 2 CD4+ T cells.

There is also the possibility that a type 2 response may be down-regulated by an incoming type 1 response. This may occur in a proliferation dependent manner. T

helper cells constantly divide to maintain effector cell numbers and it has been shown that there is inheritance of cytokine gene expression in daughter effector cells (Smale and Fisher 2002; Agarwal *et al.*, 1999). This serves to maintain polarisation of the immune response and is achieved because the chromatin remodelling that occurs in polarised cells is stable (Ansel *et al.*, 2003). However one study has shown that *in vitro* IFNγ can abrogate the proliferation of IL4 – secreting cells (Oriss *et al.*, 1997). Therefore the presence of IFNγ could down-regulate established type 2 responses by limiting the expansion of Th2 cells as they proliferate to maintain the population size.

An additional mechanism by which IFNγ can down-regulate type 2 responses is by interfering with the positive feedback loops that exist to amplify and maintain immune responses. For example the induction of T-bet by IFNγ (Lighvani *et al.*, 2001; Afkarian *et al.*, 2002) in turn induces the expression of IFNγ from T helper cells (Szabo *et al.*, 2000; Mullen *et al.*, 2001). The triggering of the IL4R complex induces GATA3 which induces the expression of IL4 (Zheng and Flavell 1997). It has been shown that cytokine feedback loops are important in the maintenance of polarised immune responses *in vitro* with the finding that continuous IFNγ is necessary to suppress IL4 secretion in individual primed type 1 cells (Zhang *et al.*, 2001). However type 2 cytokine secretion by T cells, in particular IL4 secretion, is considered to be more stable and Th cells that are committed to producing IL4 do not require further IL4 receptor engagement to continue to produce IL4 (Huang *et al.*, 1997).

On the intracellular level inhibition of positive feedback loops may occur because intracellular transcription factors involved in the transcription of IL4 or IFNγ genes can be cross-regulatory. It has been shown that the pathways involved in IL12 and IFNγ signalling can suppress GATA3 expression (Ouyang *et al.*, 2000) thereby potentially suppressing the transcription of IL4. Similarly GATA3 expression has been implicated in suppressing type 1 development (Ouyang *et al.*, 1998; Ferber *et al.*, 1999). However for interactions on the intracellular level, cells must be able to receive cytokine signals via their receptors on the surface of the cell.

It is unclear whether polarised Th2 cells express the IFNγ receptor complex at a significant level. However one study has determined that all T cells, regardless of whether they are Th1 or Th2 cells, lose the expression of the IFNγRβ chain in response to IFNγ rendering them unresponsive to IFNγ (Bach *et al.*, 1995). The ability of IL12 to interfere with an established type 2 response may be limited because although the expression of the IL12Rβ2 subunit chain is stabilised in type 1 IFNγ-producing cells (Szabo *et al.*, 1997) Th2 cells lose the ability to respond to IL-12 by down-regulating the IL12Rβ2 subunit chain (Szabo *et al.*, 1997; Rogge *et al.*, 1997). Similarly it has been shown that whilst developing Th1 cells still express the IL4 receptor complex, they have a reduced sensitivity to signalling by the IL4 receptor (Huang and Paul 1998). Although the type 1 and type 2 cytokines have the ability to cross regulate differentiation of the opposite subset, this is limited by expression of the relevant receptors. Taken together this indicates that the ability of certain type 1

cytokines, notably IFNγ and IL12, may have a limited ability to reverse individual type 2 differentiated cells.

1:4:3 Immune responses evoked by the filarial intracellular bacteria *Wolbachia* (Chapters 7 & 8).

Filarial nematodes contain intracellular bacteria known as *Wolbachia* that surround the tissues of the hypodermal cord and female reproductive tract (Henkel-Duhrsen *et al.*, 1998; Taylor *et al.*, 1999; Hoerauf *et al.*, 1999). *Wolbachia* reside in most filarial nematode species examined so far, and seem to be ubiquitous within species (Taylor and Hoerauf 1999). Phylogenetic analyses of filarial *Wolbachia* proteins indicate that the bacterial phylogeny is similar to the host nematode phylogeny (Bandi *et al.*, 1998) indicating that the relationship between *Wolbachia* and filarial nematodes is an ancient one. Removal of *Wolbachia* using tetracycline treatment leads to a multitude of effects on the nematode including impaired larval development (Bosshardt *et al.*, 1993) and infertility (Bandi *et al.*, 1999; Hoerauf *et al.*, 1999).

Antibody responses to *Wolbachia* surface protein (WSP) indicate that these intracellular bacteria do induce immune responses in filarial infection (Bazzocchi *et al.*, 2000a; Punkosdy *et al.*, 2001; Simon *et al.*, 2003). Additionally some studies indicate that the presence of *Wolbachia* in filarial nematode infection results in immune responses that encapsulate adult stages (Brattig *et al.*, 2001) and reduce the fertility of adult parasites (Taylor *et al.*, 2003). We have examined anti-WSP responses in human *Brugian* filariasis and used the *L. sigmodontis* model to

investigate if anti-*Wolbachia* immune responses play any role in filarial nematode survival.

CHAPTER 2

Materials and Methods

2:1 BUFFERS AND SOLUTIONS

All reagents used in these studies were purchased from Sigma unless otherwise stated.

2:1:1 Carbonate buffer (0.06M)

4.53% 1M solution of $NaHCO_3$

1.82% 1M solution of Na_2CO_3

The solution was adjusted to pH 9.5.

2:1:2 Citrate saline

0.85% NaCl

1.5% tri-sodium citrate.

2:1:3 Deep freeze solution for *Leishmania major* parasites

90% FCS (Gibco)

10% DMSO

2:1:4 Deep freeze solution for malaria parasites

3% Sorbitol or manitol

0.65% NaCl

28% Glycerol

All components were diluted in PBS.

2:1:5 Destain solution

10% Glacial acetic acid (BDH)

20% Methanol (Fisher)

2:1:6 Phosphate Buffered Saline (PBS)

PBS tablets were dissolved according to the manufacturer's instructions.

2:1:7 Ringers solution

27 mM KCL

27 mM $CaCl_2$,

0.15M NaCL

2:1:8 SDS Running buffer

1.84M Glycine

0.25M Tris

17.3 mM SDS

The solution was diluted 1:5 in distilled and deionised water (ddH_2O) before use.

2:1:9 Semi-defined medium

Mimimum essential medium (S-MEM) (Gibco)	140 g
(containing Earle's salt, L-glutamine, without Na_2CO_3)	
M199 medium (Gibco)	40 g
(containing Hank's solution, L-glutamine, without Na_2CO_3)	
MEM essential amino acids (Gibco)	160 ml
MEM non-essential amino acids (Gibco)	120 ml
Glucose	20 g
Hepes Buffer	160 g
MOPS buffer	100 g

(CONTINUED OVERLEAF)

Sodium pyruvate	2 g
L-alanine	4 g
L-glutamine	6 g
L-arginine	2 g
L-methionine	1.4 g
L-phenylalanine	1.6 g
L-proline	1.2 g
L-serine	1.2 g
L-taurine	3.2 g
L-threonine	7 g
L-tyrosine	2 g
Adenosine	0.2 g
Guanosine	0.2 g
Glucosamine –HCL	1 g
Folic acid	0.08 g
p-aminobenzoic acid	0.04g
Biotin	0.004g

The ingredients were dissolved in 10 litres of ddH_2O and the pH adjusted to 7.0. 40 g of $NaHCO_3$ was added and the pH corrected to 7.3. The solution filtered through a 22 µm membrane and stored at –20°C until required. The medium was diluted 1:2 with autoclaved ddH_2O before use.

2:1:10 Tail digest buffer

50 mM Tris-HCL,
2 mM NaCl
10 mM EDTA
1% SDS

Proteinase K was added immediately before use at concentration of 1 mg/ml.

2:1:11 Tris-Borate EDTA (TBE)

445 mM Tris

445 mM Boric acid

2% 500 mM solution EDTA (pH 8.0)

The solution diluted 1:5 in ddH^2O before use.

2:1:12 Tris Buffered Saline (TBS)

0.2M Tris

1.5M NaCl

The solution was adjusted to pH 7.4 and diluted 1:10 in ddH_2O before use.

2:1:13 Western blot transfer buffer

0.5X SDS running buffer

20% methanol (Fisher)

2:2 MICE

2:2:1 General mouse maintenance

The mouse strains BALB/c and C57BL/6 were purchased from Harlan or obtained from in house colonies. C57BL/6 μMT mice (Kitamura *et al.*, 1991) and C57BL/6 RAG2-/- mice (Shinkai *et al.*, 1992) were a kind gift of Professor David Gray (University of Edinburgh). C57BL/6 IL4 deficient mice (Kuhn *et al.*, 1991) (C57BL/6 IL4-/-) were purchased from Bantam and Kingman, Hull and bred in-house. With the exception of IL4-/- mice, all gene-knockout mice colonies were housed in filter top cages. For experimental work all mice were housed in filter-top cages or individually ventilated cages. All mice were given food and water *ad libitum*.

2:2:2 Mouse screening protocols

2:2:2:1 Obtaining circulating lymphocytes

Mice were screened for RAG2 deficiency by FACS analysis of tail blood. 100 µl of tail blood was collected in 50 µl heparin sulphate diluted in PBS (1000 units/ml). After a further dilution to 500 µl with PBS, the blood was overlayed onto 500 µl of Histopaque and spun at 2000 rpm for 20 minutes to remove the red blood cells. The lymphocytes were removed, washed 3 times in PBS, and stained for the B cell co-receptor molecule, CD19 and the T cell receptor accessory molecule, CD3, and analysed by Fluorescence Activated Cell Sorting (FACS).

2:2:2:2 Obtaining mouse genomic DNA

To assess the IL4 status of mice, 0.5 cm of tail tissue was digested in 100 µl tail digest buffer at 37°C for 10-14 hours. After the addition of 100 µl of ddH^2O, the proteinase K was deactivated by incubation of samples for 20 minutes at 95°C. The samples were spun down for 5 minutes at 13,000 rpm and diluted 1 in 100 with ddH_2O.

2:2:2:3 Polymerase chain reaction of mouse genomic DNA

Polymerase chain reaction (PCR) was performed on the samples. The primers used are shown in Table 2:1. 50 µl reaction mixtures containing 10 µl of tail digest were used for each reaction. The reaction mixture used for each of these reactions contained RedTaq polymerase (0.05 units / µl) and 1X PCR buffer, 0.2 µM / µl of forward

primer, 0.2 μM / μl of reverse primer (all primers synthesised by MGW Biotech) and 200 μM deoxynucleotide mix (Strategene). Components were diluted in ddH^2O. The reactions were run to the program shown in Table 2:2.

2:2:2:4 Fractionation of DNA by agarose electrophoresis

PCR products were fractionated on 1.2% agarose gels containing 0.01% ethidium bromide and diluted with 1X TBE buffer. Samples were separated using constant current (120V) for 1 1/2 hours, visualised on a transiluminator and photographed. 100 bp ladder (Gibco) was prepared according to the manufacturer's instructions and run on every gel to facilitate band sizing.

2.3 INFECTION PROTOCOLS

2:3:1 *Litomosoides sigmodontis* life cycle and experimental infection

The life cycle of *L. sigmodontis* was maintained by cyclical passage of the parasites between jirds (*Meriones unguiculatus)*, and the mite species *Ornothonyssus bacoti*, as previously described (Marechal *et al.*, 1997). Briefly the mites were fed on infected jirds with circulating microfilariae. 12-15 days later infective larvae (L3) were dissected from the mites in RPMI 1640 containing 10% FCS, 100 U / ml penicillin, 100 μg / ml streptomycin and 2 mM L-glutamine (all from Gibco) (complete RPMI). 60 L3's were collected in 0.5 ml complete RPMI and injected into the peritoneal cavity of each jird. Experimental infection was initiated with 25 L3's

PRIMER	SEQUENCE
β - actin forward	5' - TGACGGGGTCACCCACACTGTGCCCATCTA - 3'
β - actin reverse	5' - CTAGAAGCATTGCGGTGGACGATGGAGGG - 3'
IL4 forward	5' – GCTAGTTGTCATCCTGCTCTTC – 3'
IL4 reverse	5' – ACCTTCGTTGCTGTGAGGAC –3'
Neomycin reverse	5' – CCTGCGTGCAATCCATCTTG – 3'

TABLE 2:1 Primer sequences used in PCR analysis of mouse genomic DNA

PCR STAGE	Number of Cycles	Temperature	Time
1/ Initial Denaturation	**1**	**95°C**	**2 minutes**
2a/ Denaturation	**30**	**95°C**	**1 minute**
2b/ Annealing		**58°C**	**1 minute**
2c/ Extension		**72°C**	**2 minutes**
3/ Final Extension	**1**	**72°C**	**5 minutes**

Table 2:2 PCR conditions to amplify genes from mouse genomic DNA

injected subcutaneously into the right lumbar area of each mouse or else surgically implanted intraperitoneally with six live *L. sigmodontis* adult parasites removed from the peritoneal cavity of infected jirds as previously described for *B. malayi* infection (MacDonald *et al.*, 1998).

2:3:1:2 Detection of circulating *L. sigmodontis* microfilariae

The presence of circulating *L. sigmodontis* microfilariae was determined by thick circular smear of 10 µl tail blood. Smears were left to dry overnight before lysing the red blood cells with tap water. After fixing with methanol (Fisher), the smears were stained in 5% Geimsa solution for 45 minutes and examined for the presence of microfilariae by light microscopy.

2:3:2 *Leishmania* culturing and infection monitoring

The Friedlin strain of *Leishmania major* was a kind gift of Tony Aebischer (Max-Planck Institute for Infection Biology, Germany). *L. major* was stored in liquid nitrogen in *Leishmania* deep freeze solution until required. Parasites were maintained *in vivo* in BALB/c mice by sub-cutaneous injection at the base of the tail. *In vitro* maintenance was carried out in 25 cm^3 tissue culture flasks and initiated from the inguinal lymph nodes of the infected BALB/c stock mice. Parasites were cultured in semi-defined medium containing 5% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, and split 1:10 every 10 days, and not more than 4 occasions, to maintain virulence of the strain. Infections were initiated (stock and experimental) by injecting

subcutaneously or intraperitoneally with 3 x 10^6 stationary promastigotes suspended in 50 µl sterile PBS. Footpad lesions were measured weekly using digital callipers (BDH)

2:3:3 Malaria recovery and infection and monitoring

Plasmodium chabaudi (clone AS) infected red blood cells (pRBC's) were isolated from the natural rodent host (*Thamnomys rutilans*) in the Central African Republic in the period 1969 / 1970 and stored in malaria deep freeze solution in liquid nitrogen until use. pRBC's were thawed and suspended in 200 µl citrate saline. Each donor mouse was given 100 µl of the suspension intraperitoneally. Donor C57BL/6 mice, in which the parasites are passaged once before experimentation, were used in order to check parasite viability and to standardise the number of parasites used for each experimental mouse. Each experimental mouse received 1 x 10^6 pRBC's intraperitoneally diluted in 100 µl in a solution of 50% heat-inactivated calf serum and 50% Ringer solution, with 10 units of heparin / ml of mouse blood. The required volume of blood containing the correct number of pRBC's was calculated from the blood cell density and parasitaemia in the donor mice immediately before preparation.

The progress of *P. chabaudi* infection was monitored at the same time each day to reduce experimental noise. Mice were weighed, and malaria induced anaemia was measured by diluting 2 µl tail blood (removed into 2 µl capillaries and expelled by micropipette) into 80 ml's Isoton solution (Beckman Coulter). 0.5 ml of the diluted

solution was analysed on a Beckman Coulter Counter and the readings were converted to red blood cells / ml (RBC / ml) of mouse blood using a standardisation curve.

2:4 RECONSTITUTION PROTOCOL

Mice were sublethally irradiated with 400 cGy γ radiation delivered from a caesium source. 24 hours later each mouse was injected intravenously with 1 x 10^8 splenocytes prepared from naive mice as described in 2:8:1. Cells were washed and resuspended in sterile PBS. After passing through parachute silk to remove cell clumps, mice were given 100 µl of cell suspension containing 1 x 10^8 splenocytes intravenously or intraperitoneally.

2:5 VACCINATION PROTOCOLS

2:5:1 Freund's adjuvant

Antigens were diluted in PBS and mixed at a ratio of 1:1 with Freund's adjuvant with a final concentration of 200 µg / ml. The mixture was made into an emulsion by sonication on ice with repeated bursts of 20 seconds, and 20 seconds between each burst for approximately 3 minutes. Each mouse was given 100 µl sub-cutaneously (20 µg antigen). For each set of immunisations mice were given 3 doses at 4 week intervals. The first dose contained complete Freund's Adjuvant, subsequent doses were made up with incomplete Freund's Adjuvant.

2:5:2 Alum

Equal volumes of *Ls*WSP protein dissolved in PBS (at least 1 mg / ml) and 9% aluminium potassium sulphate were mixed together and the pH of the solution was adjusted to pH 7 by addition of 1M NaOH, using Phenol Red as an indicator. The mixture was left at room temperature for 30 minutes and the protein was spun down for 10 minutes at 3000 rpm. Sterile PBS was used to wash and resuspend the protein / alum precipitate. 3 doses each containing 50 μg/ml of protein / alum precipitate in 100 ul sterile PBS was administered subcutaneously to each mouse at 4 week intervals.

2:6 PROTEIN TECHNIQUES

2:6:1 Determination of protein concentration

Protein concentrations were analysed using the Bradford's dye binding method (Bradford, 1976) with Coomassie Plus Protein Assay reagent kit (Pierce). Briefly 200 μl of reagent was mixed with 20 μl of protein. Samples were read at 595 nm. The blue colour change induced by the protein sample was compared to that induced by a standard curve of known quantities bovine serum albumin (BSA) standard (Pierce).

2:6:2 Separation of proteins by SDS – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were diluted 1:1:1 with ddH^2O and a mixture of equal volumes of 1.25M DTT and 3X SDS loading buffer (both New England Biolabs). The diluted samples were reduced by incubating at 95°C for 5 minutes . Samples were run on precast gels

with an agarose gradient of 4-12% (Invitrogen). Gels were run at 120V for 1 hour in MES buffer (Invitrogen).

2:6:3 Visualisation of protein separation by SDS-PAGE

2:6:3:1 Coomassie staining

Separated proteins were stained by immersing gels in 0.5% Coomassie stain overnight. Acrylamide gels were destained in destaining solution and air dried between two sheets of gel drying film.

2:6:3:2 Silver staining

Gels were fixed for 2 hours in a solution containing 30% ethanol (BDH) and 10% glacial acetic acid (BDH). Gels were prepared for staining by incubating overnight in a solution containing 30% ethanol, 0.5M sodium acetate (3 hydrate) ($CH_3COONa.3H_2O$), 2% of 25% gluteraldehyde (BDH) and 20 mM sodium thiosulphate ($Na_2S_2O_3$). After washing 3 times in ddH^2O separated proteins were stained for 1 hour in 0.1% silvernitrate ($AgNO_3$) solution containing 0.054% formaldehyde (BDH). Gels were washed briefly in ddH^2O and developed in 2.5% sodium carbonate (Na_2CO_3) solution with 0.025% formaldehyde. The reaction was stopped with a solution containing 0.05M EDTA and the gels air-dried between two sheets of gel drying film in glycerol solution (2% glycerol and 7% glacial acetic acid).

2:6:4 Western blotting

Parasite extracts were separated by SDS PAGE and transferred to a nitrocellulose membrane (Amersham) at 0.8 mA /cm^2 membrane for 1 hour. The efficiency of the transfer was assessed by a brief staining of the membrane in Ponceau-S. After blocking for 1 hour in blocking solution (1X TBS containing 0.05% Tween (TBST) and 5% skimmed milk powder (Marvel) the blots were incubated overnight at 4°C with experimental mouse sera diluted 1:10 in blocking solution. Blots were washed in TBST for 1 hour at room temperature, changing the wash solution every 10 minutes and then incubated with secondary antibody at room temperature for 1 hour. Secondary antibody conjugated to HRP was diluted according to Table 2:4 in blocking solution. The washing step was repeated and ECL Plus substrate (Amersham) was added according to the manufacturer's instructions. Blots were developed onto ECL hyperfilm (Amersham).

2:6:5 Generation of recombinant *Wolbachia* Surface Proteins

2:6:5:1 Cloning of *Brugia malayi* WSP (*Bm*WSP) and *L. sigmodontis* WSP (*Ls*WSP) homologues

B. malayi WSP (*Bm*WSP) and *L. sigmodontis Ls*WSP homologues were cloned by PCR amplification of a 652 bp fragment from genomic DNA using the degenerate WSP primers (as described in Bazzocchi *et al.*, 2000b). Both gene fragments were cloned into pET29b (Novagen) and expressed as hexamer-his-tagged proteins in 500 ml *Escherichia coli* BL21(λDH3) cultures using standard protocols. Both fragments

have been deposited into Genbank (accession numbers AJ252061 and AF409112 respectively). The generation of *Brugia pahangi* ladder protein has been described previously by Paxton *et al.* (1993).

2:6:5:2 Purification of *Bm*WSP and *Ls*WSP homologues

E. coli cultures were centrifuged at 10,000 *g* for 10 minutes and the supernatant discarded. The cultures were divided into 100 ml samples. Each sample was washed in 40 mls cold binding buffer (Novagen) and resuspended in 20 ml binding buffer containing 100 μg/ml lysozyme for 1 hour at room temperature. The suspensions were sonicated on ice as described in 2:5:1. After centrifugation at 10,000 *g* for 10 minutes, samples of the supernatants and inclusion bodies in the pellets were analysed by SDS-PAGE to determine the solubility of the recombinant protein. For insoluble proteins the pellets were each resuspended in 20 ml binding buffer containing 6M Urea and incubated on ice for 1 hour to dissolve the proteins. Insoluble material was removed by centrifuging at 16,000 *g* for 30 minutes and the supernatant filtered through a 0.45 μm filter.

The recombinant his-tagged proteins were purified by affinity chromatography using a separation column with a settled bed volume of 2.5 ml of resin (Novagen). The resin was charged and prepared for protein separation according to the manufacturer's instructions. Protein separation was undertaken at 10 column volumes / hour using a peristaltic pump. A His-Bind Buffer Kit was used at all stages (Novagen). The purity

of the protein was determined by silver stained SDS-PAGE gels and the purified protein was dialysed into sterile PBS before use.

2:7 PARASITE ANTIGEN PREPARATION

2:7:1 *L. sigmodontis* and *B. malayi* extracts

Somatic extracts of L3, adult or Mf parasites were prepared by homogenisation and, for L3 and Mf preparations, sonication of these parasites in PBS using the sonication protocol described in 2:5:1. Unless otherwise stated somatic extracts were centrifuged at 1000 x *g* for 20 minutes and the pellet discarded. The extract was stored at -20^0C until required.

2:7:2 *L. major* extract

Stationary *L. major* promastigotes were suspended in sterile PBS at a concentration of 1×10^{10}. Parasites were lysed by freezing and thawing the suspension 3 times. The extract was stored at –20°C until required.

2:7:3 Lysed *P. chabaudi* parasitised red blood cell extract

Mice with a parasitaemia of at least 10 % were bled from the brachial artery into lithium heparin vacutainers (BDH). The blood was centrifuged at 400 *g* for 15 minutes and the pellet lysed with 5 ml of ammonium chloride red blood cell lysis buffer (Sigma). After a 5 minute incubation, the solution was diluted 1:2 with Dulbecco's

Modified Eagles's Medium (DMEM), centrifuged again at 400 *g* for 15 minutes and the pellet discarded. The supernatant was stored at –20°C until required.

2:8 CELL CULTURE

Cells were removed from lymph nodes or spleen by mashing through a cell strainer and suspended in DMEM supplemented with 10% foetal calf serum, 100 U / ml penicillin, 100 μg / ml streptomycin and 2 mM L-glutamine (all from Gibco) (complete DMEM). Red blood cells were removed using red blood cell lysis buffer and the white blood cells washed in complete DMEM twice. Cell were resuspended in DMEM supplemented with 0.5% mouse serum, 100 U / ml penicillin, 100 μg / ml streptomycin and 2 mM L-glutamine and plated at 1 x 10^6 splenocytes / well for analysis. In all cases antigenic stimulation was carried out at a final concentration of 10 μg / ml with the exception of Con A which was carried out at a final concentration of 1 μg / ml. Cells were incubated for 72 hours at 37^{0}C + 5% CO_2. Supernatants were removed and frozen at –20^{0}C until required and cells were pulsed for a further 18 hours (2 rounds of cell division) with tritiated thymidine (Amersham). Cells were harvested onto scintillation sheets and thymidine incorporation measured on a scintillation counter.

2:9 CYTOKINE DETECTION

2:9:1 Cytokine ELISA

96 well ELISA plates (Nunc Maxisorp) were coated with the desired amount and

concentration of capture antibody diluted in carbonate buffer (see Table 2:3) and left overnight at 4°C. After blocking for 1 1/2 hours at 37°C with 100 µl of 5% skimmed milk powder (Marvel) diluted in carbonate buffer, 50 µl of sera samples were loaded onto each well in duplicate. Standard curves of two- fold serial dilutions of recombinant mouse cytokines were constructed in duplicate. Plates were left overnight at 4°C. Biotinylated detection antibody was diluted in TBST with 0.5% FCS (Table 2:3) and added to each well. The plates were left at 37°C for 1 1/2 hours. Following an additional incubation with Extravidin-Alkaline Phosphatase diluted 1:25,000 in TBST with 0.5% FCS, or Streptavidin-HRP (Amersham) diluted 1:8000 with 0.5% FCS for 30 minutes, plates were developed with 100 µl of p-Nitrophenyl Phosphate (pNPP) and read at 405 nm, or Sure Blue TMB system (Kirkegaarde and Perry Laboratories, Inc.), and read at 620 nm, respectively. Plates were washed 5 times with TBST in between each wash, with an additional wash on ddH^2O before adding the substrate. All capture and detection antibodies were purchased from Pharmingen.

Cytokine	Capture Antibody Used	Biotinylated Antibody Used	Initial Value of Standard Curve
IFNγ	R4-6A2, 50 µl, 3 µg/ ml	XMG1.2, 50 µl 0.5 µg / ml	500 U / ml
IL4	11B11, 100 µl, 4 µg / ml	BVD6-24G2, 100 µl, 0.5 µg / ml	4 ng / ml
IL10	JES5-2A5, 100 µl, 5 µg / ml	SXC-1, 100 µl, 1 µg / ml	5 ng / ml

TABLE 2:3 Mouse cytokine ELISA antibody pairs.

2:9:2 IL4 and IFNγ ELISPOT

ELISPOT plates (Millipore) were pre-wetted with 100 µl of 100% ethanol and washed once with 200 µl of autoclaved ddH^2O. 50 µl of capture antibody, diluted to 15 µg / ml in sterile carbonate buffer, was added to each well (capture antibodies as in Table 2:3). The plates were left overnight at 4°C wrapped in foil and then washed once with 200 µl of sterile TBST. 150 µl of 2% skimmed milk powder (Marvel) diluted in sterilised carbonate buffer, was added to each well. Plates were left at 37°C for at least 2 hours and then washed twice with 200 µl sterile TBST per well. Following a further wash with 200 µl sterile PBS per well to remove traces of Tween, 50 µl of cell suspensions containing 5×10^5 cells (splenocytes) or 3×10^3 cells (lymph node cells) in DMEM supplemented with 100U / ml penicillin, 100 µg / ml streptomycin, 2mM L-glutamine (all from Gibco) and 0.5% mouse serum were added to each well. Antigenic stimulation was carried out as for proliferation assays (2:8) and the plates were incubated for 72 hours at 37°C + 5% CO_2 wrapped in aluminium foil.

After incubation plates were washed 5 times in TBST followed by 1 wash of ddH^2O to lyse any remaining cells. 50 µl of biotinylated antibody diluted in TBST with 0.5% FCS was added to each well (antibody's as in Table 2:3- IFNγ – 1 µg / ml; IL4 - 0.1 µg / ml) and incubated for 2 hours at 37°C. After washing 5 times in TBST, 50 µl Extravidin AP diluted 1 : 25,000 in TBST supplemented with 0.5% FCS was added to each well. Plates were washed 5 times in TBST and once in ddH^2O to remove traces of Extravidin AP. 100 µl of syringe filtered (0.45 µm) BCIP / NBT substrate (Moss

substrates, Inc.) was added to each well to develop spots. Upon spot development the plates were washed in ddH^2O and left to dry. Capture and detection antibodies were purchased from Pharmingen. Plates were read by an ImmunoSpot analyser (Cellular Technology Ltd) and analysed using ImmunoSpot software.

2:10 ANTIBODY DETECTION

2:10:1 Antigen - specific IgG isotypes

96 well ELISA plates (Nunc Maxisorp) were coated with 0.5 μg antigen per well diluted in carbonate buffer and left overnight at 4°C. After blocking with 100 μl of 1% skimmed milk powder (Marvel) diluted in carbonate buffer for 90 minutes at 37°C, 50 μl of sera samples diluted in PBS with 0.5% Tween (PBST) were loaded onto each well in twofold serial dilutions of 1 in 100 to 1 in 3200 (human samples) or of 1:100 to 1:400 (mouse samples). The optical density values for each sample were plotted against each range of dilutions as depicted in Fig. 2:1. A dilution for each group of samples (mouse isotypes and human total IgG) was chosen whereby all samples fell in the linear range of the curve. For mouse IgG1, IgG2b and IgG3 this dilution was 1:200, for mouse IgG2a this dilution was 1:100 and for human total IgG this dilution was 1:400. Each sample was plated in duplicate and left for 90 minutes at 37°C. Antibodies were detected with 50 μl of peroxidase-conjugated detection antibodies diluted in PBST (Table 2:4). After a further 90 minute incubation step at 37°C plates were developed with 50 μl of 2,2'-azinodi(ethylbenzthiazo-line-6-sulfonate) (ABTS, Kirkegaarde and Perry Laboratories, Inc.) and read at 405 nm.

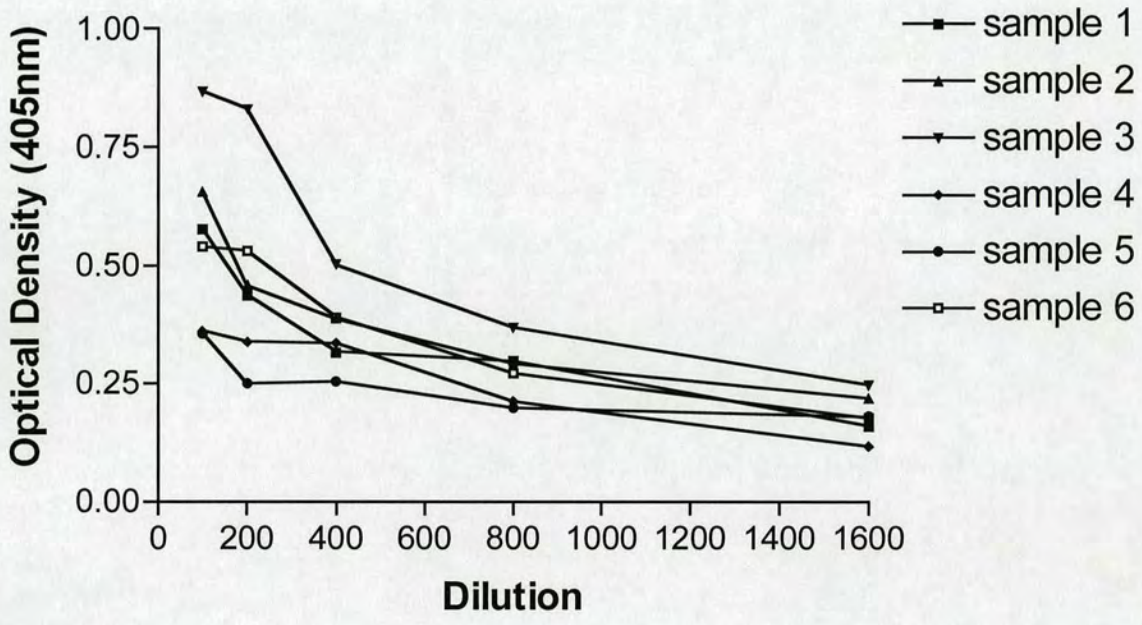


Fig. 2:1 The dilution of serum samples in antibody ELISA. The graph depicts 6 different samples diluted two fold from 1:100 to 1:1600. Based on this graph a dilution of 1:200 would be selected for analysis because at this dilution most of the samples fall within the linear range of the curve. 1:100 was not chosen because sample 6 has reached the maximum optical density at 1:200 and therefore would be under-represented relative to the other samples at a dilution of 1:100.

2:10:2 Polyclonal IgE determination

IgE sandwich ELISA was carried out using the cytokine ELISA protocol as outlined in 2:9:1. Plates were coated with 100 µl of IgE capture antibody (clone R35-72, Pharmingen) at a concentration of 2 µg / ml. Sera were added at dilutions of 1:10 and 1:20. A standard curve was created in duplicate using two- fold serial dilutions beginning at 20 µg / ml of purified mouse IgE, κ monoclonal isotype standard (Pharmingen). 100 µl of biotinylated detection antibody (clone R35-118) (Pharmingen) was added at a concentration of 2 µg / ml.

SPECIFICITY	ISOTYPE	DILUTION	SUPPLIER
Goat anti-MOUSE	Total IgG - HRP	1:1000	Biorad
Goat anti-MOUSE	IgG1 - HRP	1:6000	Southern Biotechnology Associates Inc.
Goat anti-MOUSE	IgG2a – HRP (BALB/c)	1:4000	Southern Biotechnology Associates Inc.
Goat anti-MOUSE	IgG2a - HRP (C57BL/6)	1:200	Southern Biotechnology Associates Inc.
Goat anti-MOUSE	IgG2b - HRP	1:4000	Southern Biotechnology Associates Inc.
Goat anti-MOUSE	IgG3 - HRP	1:1000	Southern Biotechnology Associates Inc.
Rabbit anti-HUMAN	Total IgG - HRP	1:6000	Dako

Table 2:4 Detection antibody's used in antibody ELISA's and Western Blotting

2:11 CELL IDENTIFICATION BY FACS

Fluorescent Activated Cell Sorting (FACS) was used to identify cells within various populations. Equal cell numbers (between 1 x 10^5 and 1 x 10^6) were used in any one

experiment. Cells were washed in PBS containing 2% FCS (FACS buffer) and incubated with 50 µl α-CD16 antibody (Pharmingen) diluted 1:50 in FACS buffer for 20 minutes at 4°C. After 20 minutes staining antibodies (Table 2:5, all Pharmingen) diluted in FACS buffer were added and the cells incubated at 4°C for a further 30 minutes. Cells were washed twice in FACS buffer and fixed in a solution of 2% paraformaldehyde before being read on a Becton Dickenson FACScan. Data was analysed using Becton Dickenson Cell-Quest Software. Dead cells were gated out of the analysis.

SURFACE MARKER	LABEL	CLONE	DILUTION
CD3	PE	17A2	1:200
CD4	PE	GK1.5	1:200
CD8a (Ly-2)	Cy-Chrome	53-6.7	1:100
CD19	FITC	ID3	1:200
Ra IgG2b, κ isotype standard	PE	A95-1	1:200
Rat IgG2a κ Isotype standard	Cy-Chrome FITC	R35-95	1:200

Table 2:5 Antibodies used in FACS analysis

2:12 IMMUNOHISTOCHEMISTRY

2:12:1 Generation of nematode sections

Encysted *L. sigmodontis* adult parasites were recovered from the thoracic cavity of mice at 60 days post-infection. Parasites were embedded in Cryo-M-Bed embedding compound (Bright Instrument Company Ltd.) and stored at –80°C until sectioning. 5

μm frozen sections were cut on a cryostat and placed on glass slides. The sections were fixed in methanol, air-dried and stored at –20°C until required.

2:12:2 Staining of nematode sections for neutrophils

Sections were washed in PBS for 10 minutes. 50 μl of α-CD16 antibody (Pharmingen) diluted 1:50 in PBS was added to each section and left in a humid environment at room temperature for 2 hours. An antibody specific for mouse neutrophils (Clone 7/4, Caltag) tagged with PE was diluted 1:200. 50 μl was added on top of the α-CD16 antibody. The sections were left for 1 hour, again in a humid environment at room temperature. As a control, some sections were incubated with a PE- labelled anti-rat control (Clone R35-95, Pharmingen). The sections were washed thoroughly for 1 hour in PBS, with the wash solution changed every 10 minutes. The sections were covered with DPX mount (BDH) and a large coverslip and visualised by fluorescence microscopy.

2:13 STATISTICAL ANALYSIS

2:13:1 Non-parametric analysis

Non-parametric analysis was used to analyse data with less than 10 samples or when the data to be analysed did not conform to the assumptions of parametric testing (see 2:13:2). A Mann-Whitney U-test (Minitab, Inc) was used to test for differences between two groups. A Kruskal Wallis test (Minitab, Inc) was used to test for differences between three or more groups. For both tests a P value < 0.05 was

considered statistically significant. In the case of a statistically significant result from a Kruskal Wallis test, subsequent testing with Dunn's Pairwise Multiple comparison test (Prism Inc.) was used to identify pairwise differences. Two-tailed tests were used unless otherwise stated.

2:13:2 Parametric analysis

Parametric testing was used when there were 10 or more data points per group, the data showed a normal distribution (Anderson Darling test for normality, $P>0.05$, Minitab, Inc.) and the variances of the groups tested were homogenous (Bartlett's test for homogeneity of variance, $P>0.05$, Minitab Inc.). In cases where datasets did not conform to the latter two assumptions, transformation of the data was attempted (as detailed in each analysis). Datasets that did not meet all three criterion detailed above were non-parametrically tested.

Differences between two datasets were tested using Student's t-test (Minitab Inc.). Differences between three or more datasets were tested using one-way analysis of variance (ANOVA) (Minitab Inc.). In the case of a statistically significant result from a one-way ANOVA, subsequent testing with Tukey's Pairwise Multiple comparison test (Prism Inc.) was used to identify pairwise differences. Unless otherwise stated all tests were two-tailed.

2:13:3 Regression analysis

General linear modelling (GLM) (Minitab Inc.) was used to test for associations between two or more variable parameters between experimental groups. To ensure the data in the model conformed to the assumptions of a GLM, the residuals of each model (the differences between the observed and predicted values of each data point) were checked for homogeneity of variance and normality of distribution (Grafen and Hails 2002). Attempts were made at data transformation (as detailed for each analysis) when residuals from the maximal model did not meet with these assumptions.

Logistic regression analysis was undertaken to test for differences between the proportion of animals with live infections at the end of experiments, and the proportion of animals with circulating microfilariae.

2:13:4 Meta-analysis

Due to the small numbers of animals used in each experimental group, and the variability within groups, some data were analysed together. General Linear Modelling was used for meta-analysis as described in 2:13:3 to remove experimental effects. Meta-analysis was used only when there were no interactions observed between experiment and the variable being tested. Unless otherwise stated P values from this analysis are from models including experimental effects.

CHAPTER 3

The Role of IL4 in Filarial Infection

(This work was done in conjunction with Dr Laetitia Le Goff)

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3:1 Introduction

It has long been recognised that the majority of nematode infections elicit a strong type-2 response. Certainly this is the case for filarial infections in both humans and mice (for review see Ottesen, 1992; Lawrence and Devaney, 2001). Such a response is typified, and is also driven, by the production of interleukin 4 (IL4). This response is in contrast to that generated by most microbial pathogens, which generally evoke a type-1 immune response. The reason why helminths evoke a type 2 response is unknown, although in mouse models of filariasis IL4 transcripts in cells of the immune system can be detected within 24 hours of infection (Osborne and Devaney, 1998; Balmer and Devaney, 2002) indicating that this is a very rapid response.

It is difficult to determine the role of IL4 in human filarial infection as it is ubiquitously expressed in different clinical groups displaying a spectrum of disease symptoms and harbouring an unknown quantity of nematodes (Ottesen 1992; King *et al.*, 1993; Dimock *et al.*, 1996). There are no good mouse models for the pathology of filarial infection. However mouse models can be used as a tool to examine the role of this type-2 cytokine in resistance and susceptibility to filarial nematode infection.

Infection of mice with the rodent filarial nematode *L. sigmodontis* has revealed differences in the resistance and susceptibility of different strains of mice to this parasite. The only fully susceptible strain is the BALB/c mouse, with most other strains of mice being resistant to parasite development (Petit *et al.*, 1992). One study has demonstrated that IL4 has limited effects on the establishment of *L. sigmodontis* in BALB/c mice (Volkmann *et al.*, 2001). This has also been observed in infections of *B. pahangi* (Devaney *et al.*, 2002). However strikingly both of these studies revealed a role for this cytokine in control circulating microfilariae (Mf).

Consistent with studies of *L. sigmodontis* infections of resistant B10D2 mice (Petit et al., 1992; Marechal et al., 1996) circulating microfilariae have never been observed in *L. sigmodontis* infections of C57BL/6 mice, even when some nematodes are recovered from the thoracic cavity (Le Goff *et al.*, personal communication). It is unknown whether this occurs because there are not enough nematodes present to mate, whether the nematodes that do establish are not fecund, or whether Mf are produced but are cleared in these infections. One aim of this study was to determine whether we could reverse the amicrofilaraemia of C57BL/6 mice by infecting IL4 deficient animals with *L. sigmodontis* since IL4 is important in the control of microfilaraemia in BALB/c mice.

In these studies we observed microfilaraemia in IL4 deficient C57BL/6 mice that was comparable to susceptible BALB/c mice. However to our surprise, we also observed that IL4 appears to be an important immunological factor mediating resistance to the

establishment of an *L. sigmodontis* infection in C57BL/6 mice. C57BL/6 IL4-/- mice harboured the same number of live nematodes at 60 days post-infection as BALB/c susceptible mice. Therefore it was unclear whether the reversal of amicrofilariaemia in C57BL/6 mice was due to the removal of IL4 dependent mechanisms that influence Mf production and clearance, or whether it was due to an increase in the number of adult filarial nematodes that were able to survive and mate. Nematode establishment and circulating microfilaraemia in BALB/c and C57BL/6 IL4 -/- mice were associated with an increase in IFNγ secretion from splenocytes stimulated with adult *L. sigmodontis* antigen when compared with C57BL/6 wild type mice. Furthermore we recovered some adult parasites from C57BL/6 resistant mice that were encysted in a granulomatous material and this phenomenon was not observed in infections of either BALB/c or C57BL/6 IL4-/- mice.

3:2 Results

3:2:1 *IL4 protects C57BL/6 mice against the establishment of* L. sigmodontis *infection.*

C57BL/6 mice are largely resistant to infection with *L. sigmodontis* (Petit *et al.*, 1992). In our experiments, less than 1% of the infective stages of *L. sigmodontis* (L3) injected survived until day 60 post-infection (Fig. 3:1A). In contrast an average of 9% of larvae were able to survive until day 60 post-infection in C57BL/6 mice deficient in IL4 (C57BL/6 IL4-/-) (Fig. 3:1A). The recovery rate in C57BL/6 IL4-/- mice was similar to BALB/c susceptible mice indicating that IL4 is responsible for resistance in

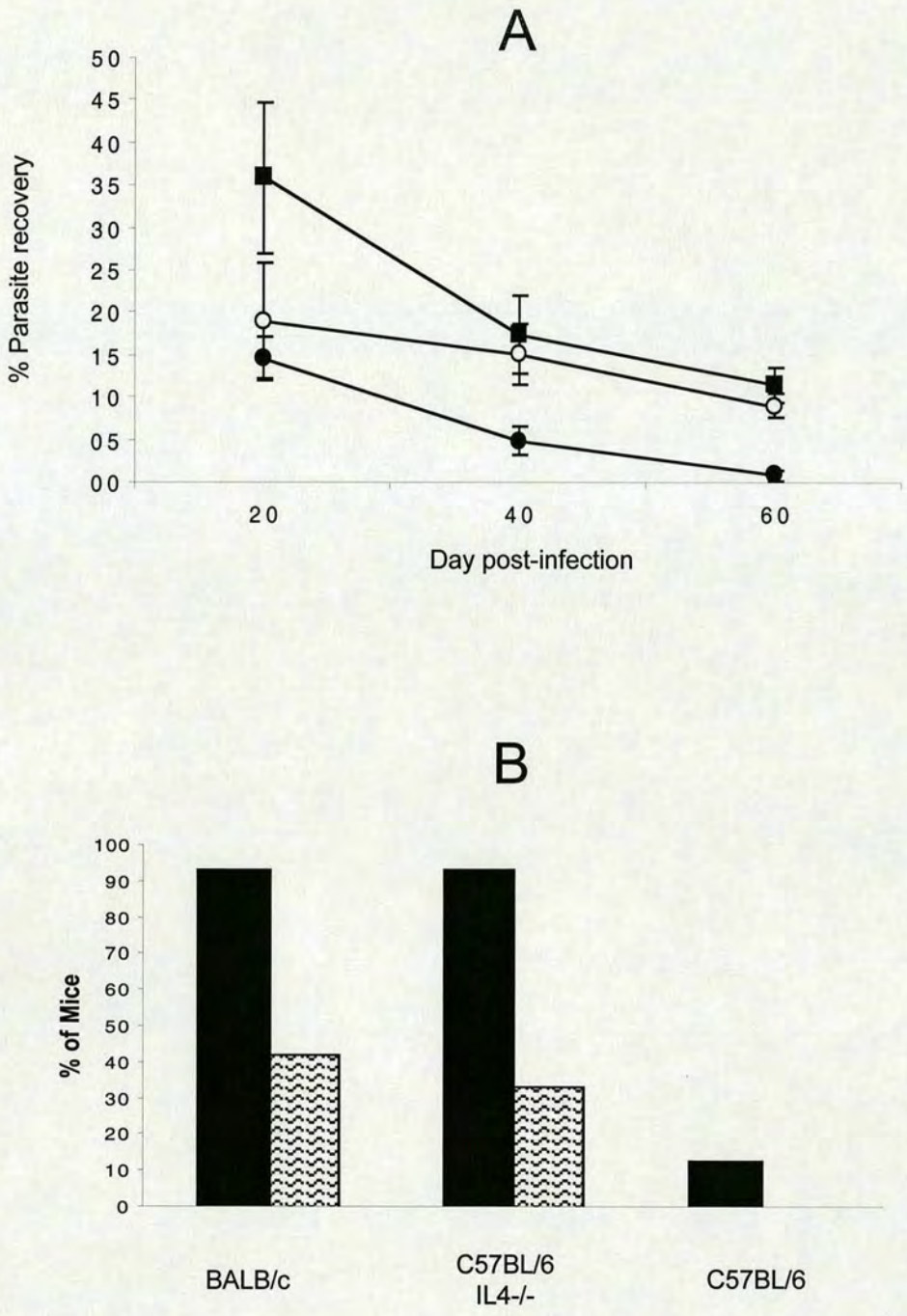


Figure 3:1. Parasitology of C57BL/6 mice, C57BL/6 IL4-/- mice and BALB/c mice at 20, 40 and 60 days post-infection. (A) The percentage(%) of adult parasite recovery from the initial L3 innoculum in C57BL/6 mice (black circles) and C57BL/6 IL4-/- mice (white circles) at days 20, 40 and 60 post-infection (p.i.). BALB/c mice (black squares) are shown for comparison. Values shown are from different experiments at each time point (Day 20 p.i.: two experiments, Day 40 p.i.: four experiments and Day 60 p.i: four experiments), and each experiment contained at least two different genotypes of mice, one experiment with all genotypes of mice was performed at each time point. Error bars show the SEM of percentage recovery within genotypes. (B) The % of infected mice with active filarial infection at day 60 from the 4 experiments shown in (A). Black bars represent the % of mice harbouring one or more live adult parasites. Dashed bars represent the % of mice that were found to have circulating microfilariae.

C57BL/6 mice. Indeed the % of mice harbouring parasites at this time point was around 90% for both C57BL/6 IL4-/- and BALB/c mice compared to 10% of C57BL/6 mice (Fig 3:1B).

Most parasite attrition occurred in the first 20 days after infection in all strains of mice tested (Fig. 3:1A). Additionally there was a large variation between mice of each genotype in the % of larvae that were able to migrate to the thoracic cavity at this time point. In general BALB/c mice had twice the amount of parasites in the thoracic cavity compared to C57BL/6 mice at 20 days post-infection (Fig. 3:1A). This difference was not found to be statistically significant (Tukey's pairwise multiple comparison test $P>0.05$) although this trend has been observed in other studies (Marechal *et al.*, 1996).

C57BL/6 IL4-/- mice had a parasite recovery rate that was more similar to C57BL/6 mice than to BALB/c mice at day 20 post-infection indicating that IL4 is not involved in the intrinsic increased resistance of C57BL/6 mice to early infection. At day 40 post-infection the C57BL/6 IL4 deficient mice recovery rate was more similar to the BALB/c mice than C57BL/6 mice suggesting that IL4 mediates parasite damage between days 20 and 40 post-infection, when the L4 stages moult to become adults (Marechal *et al.*, 1996). From days 40 to 60 post-infection the parasite attrition rate appears to be similar in all three genotypes of mice tested (Fig. 3:1A), again suggesting that IL4 mediates resistance in C57BL/6 mice between days 20 and 40 post-infection. The only statistical differences in recovery rate between the three genotypes of mice were between C57BL/6 mice and BALB/c mice at days 40 and 60

post-infection (Tukey's pairwise multiple comparison test $P<0.05$ at day 40 PI and $P<0.01$ at day 60 PI).

Adult female parasites taken from B10D2 mice, another strain of mouse that is resistant to the establishment of infection with *L. sigmodontis*, have never been found to be gravid, and circulating Mf have never been observed in these mice (Petit *et al.*, 1992; Marechal *et al.*, 1996). Likewise in these experiments we did not find any circulating Mf in C57BL/6 mice. 42% of BALB/c mice became microfilaraemic (Fig. 3:1B) which is similar to previously published studies (Petit *et al.*, 1992; Marechal *et al.*, 1996) and interestingly we also observed circulating Mf in C57BL/6 IL4-/- mice. 33% of C57BL/6 IL4-/- mice became microfilaraemic (Fig. 3:1B).

3:2:2 Splenocytes from susceptible C57BL/6 IL4-/- and BALB/c mice, but not resistant C57BL/6 mice, secrete IFNγ in response to L. sigmodontis *at 60 days post-infection.* To investigate possible immunological mechanisms that correlate with resistance and susceptibility to *L. sigmodontis*, we decided to examine the cytokines secreted against adult parasites in the three genotypes of mice tested in these experiments. We looked at splenocytes stimulated with adult *L. sigmodontis* homogenate from mice infected with parasites for 60 days to get a snapshot of the immune response when BALB/c mice and C57BL/6 IL4-/- mice harbour similar numbers of parasites and have circulating microfilariae. As a measure of type 1 and type 2 responses we measured IFNγ and IL4 respectively. Because C57BL/6 IL4-/- mice cannot secrete IL4, this cytokine could not be used as an indicator of anti-parasite type-2 like responses in this

genotype of mice. We also measured interleukin 10 (IL10) which is sometimes considered another indicator of type 2 responses, although it is also an indicator of type 3 regulatory responses (Akdis and Blaser 2001).

At 60 days post-infection both BALB/c susceptible and C57BL/6 resistant mice secreted IL4 in response to adult *L. sigmodontis* (Fig. 3:2A) although unlike the previous study comparing B10.D2 and BALB/c mice (Marechal *et al.*, 1997) the amount of cytokine was comparable between the resistant and susceptible strains of mice (Tukey's pairwise multiple comparison test $P>0.05$). As expected stimulated splenocytes from C57BL/6 IL4-/- mice secreted no parasite-specific IL4. The splenocytes from both susceptible strains of mice, but not C57BL/6 resistant mice, secreted IFNγ in response to adult *L. sigmodontis* (Fig. 3:2B) although the level of IFNγ was much lower than that induced in the spleen by the type 1 response-inducing pathogen *Leishmania major,* which is in excess of 500U/ml for the same number of splenocytes (Lamb *et al.*, unpublished observation). Nevertheless secretion of parasite-targeted IFNγ (or the presence of a type 1 response) correlates with the presence of adult parasites and circulating microfilariae in these genotypes of mice.

All three groups of mice secreted some IL10 in response to *L. sigmodontis* parasites (Fig. 3:2C). However the amount of IL10 secreted by C57BL/6 mice was barely above that of control naive mice. Notably C57BL/6 IL4-/- mice secreted less IL10 than BALB/c mice despite harbouring the same number of adult parasites at this time point, although this was not statistically significant (Kruskal Wallis $P=0.08$).

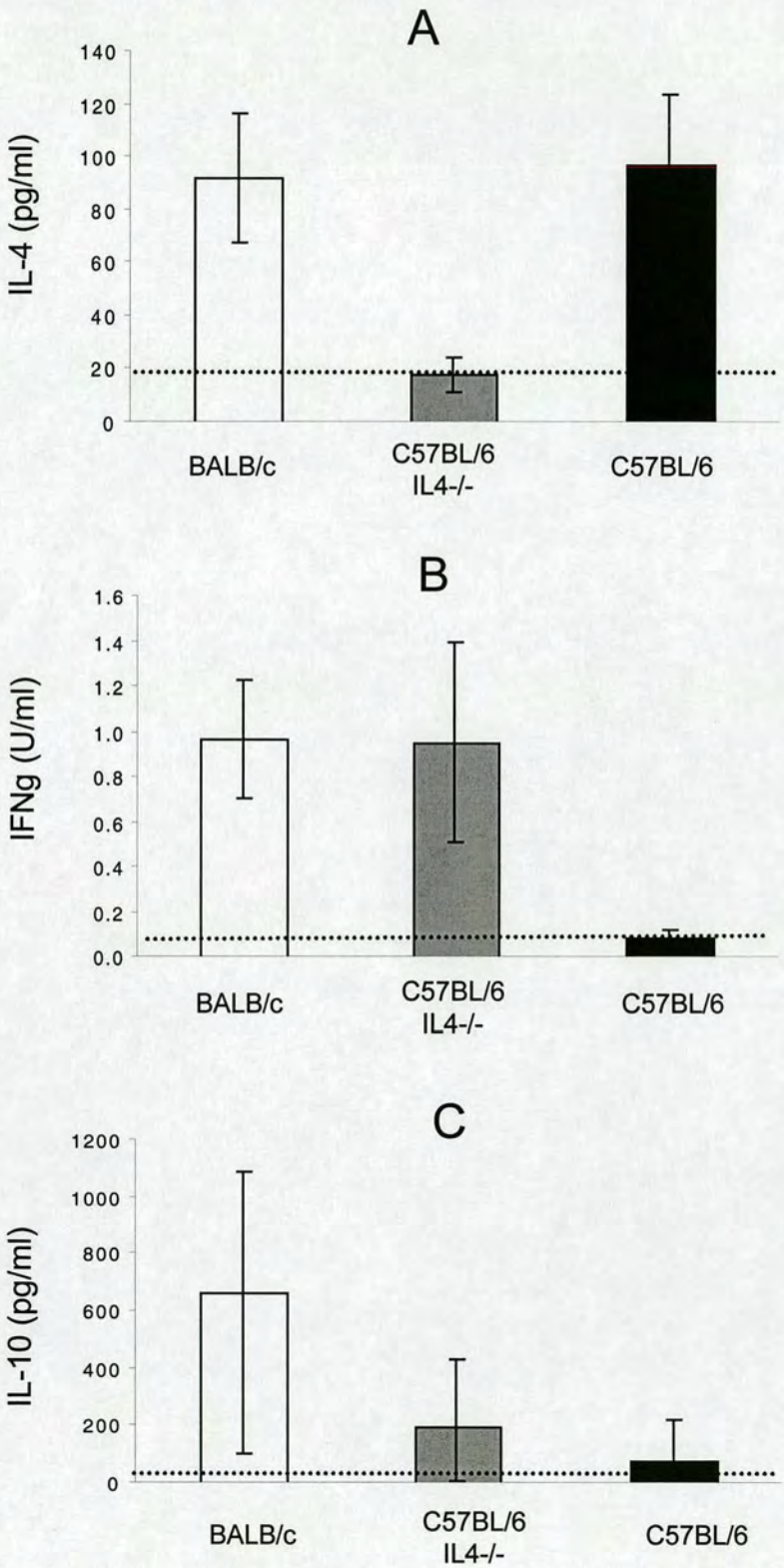


Figure 3:2. Cytokine secretion by splenocytes in response to *L. sigmodontis* adult homogenate at 60 days post-infection. IL4 (A), IFNγ (B) and IL10 (C) are shown. The white bars represent BALB/c mice, the grey bars represent C57BL/6 IL4-/-mice and the black bars represent C57BL/6 mice. The error bars in A and B represent the SEM for each group. The error bars in C represent the maximum and minimum values for each group.The dashed lines represent the average amount of cytokine secreted by splenocytes from naive animals. Three experiments are represented by A and B. One experiment is represented in C.

3:2:3 Susceptible BALB/c mice mount early IgG1 and IgG2a antibody responses to L. sigmodontis.

As we observed differences in splenocyte cytokine secretion against *L. sigmodontis* between the three genotypes of mice, we decided to analyse the antibody isotypes produced in response to infection. Antibodies reflect cytokine driven B cell responses over time and therefore may be more representative of the cumulative response to *L. sigmodontis* infection than single time-point cytokine analysis. Overall in these experiments we did not observe substantial levels of *L. sigmodontis* specific antibody until day 40 post-infection (Fig 3:3). This could be due to the fact that *L. sigmodontis* homogenate made from the adult stage, rather than the L3 stage, was used as an antigen and the parasites do not moult into adults until day 20 post-infection (Marechal *et al.*, 1996). Nevertheless at day 20 post-infection resistant BALB/c mice appear to produce low levels of IgG1 and IgG2a that appear to be absent in C57BL/6 IL4-/- and C57BL/6 wild type mice (Figs. 3:3A and 3:3B).

At day 40 post-infection, when the parasite recovery rate in C57BL/6 IL4-/- mice is similar to that in BALB/c mice, C57BL/6 IL4-/- mice produce less anti-parasite IgG1 ,but more IgG2a, than BALB/c mice (logarithmically transformed data, Tukey's pairwise multiple comparison test $P<0.01$ in both cases) (Figs. 3:3A and 3:3B). At day 60 post-infection, C57BL/6 IL4-/- mice produce high levels of IgG2a and IgG2b in comparison to both C57BL/6 mice and BALB/c mice (Figs. 3:3B and 3:3C). Additionally C57BL/6 mice appear to produce a higher level of IgG2a when compared

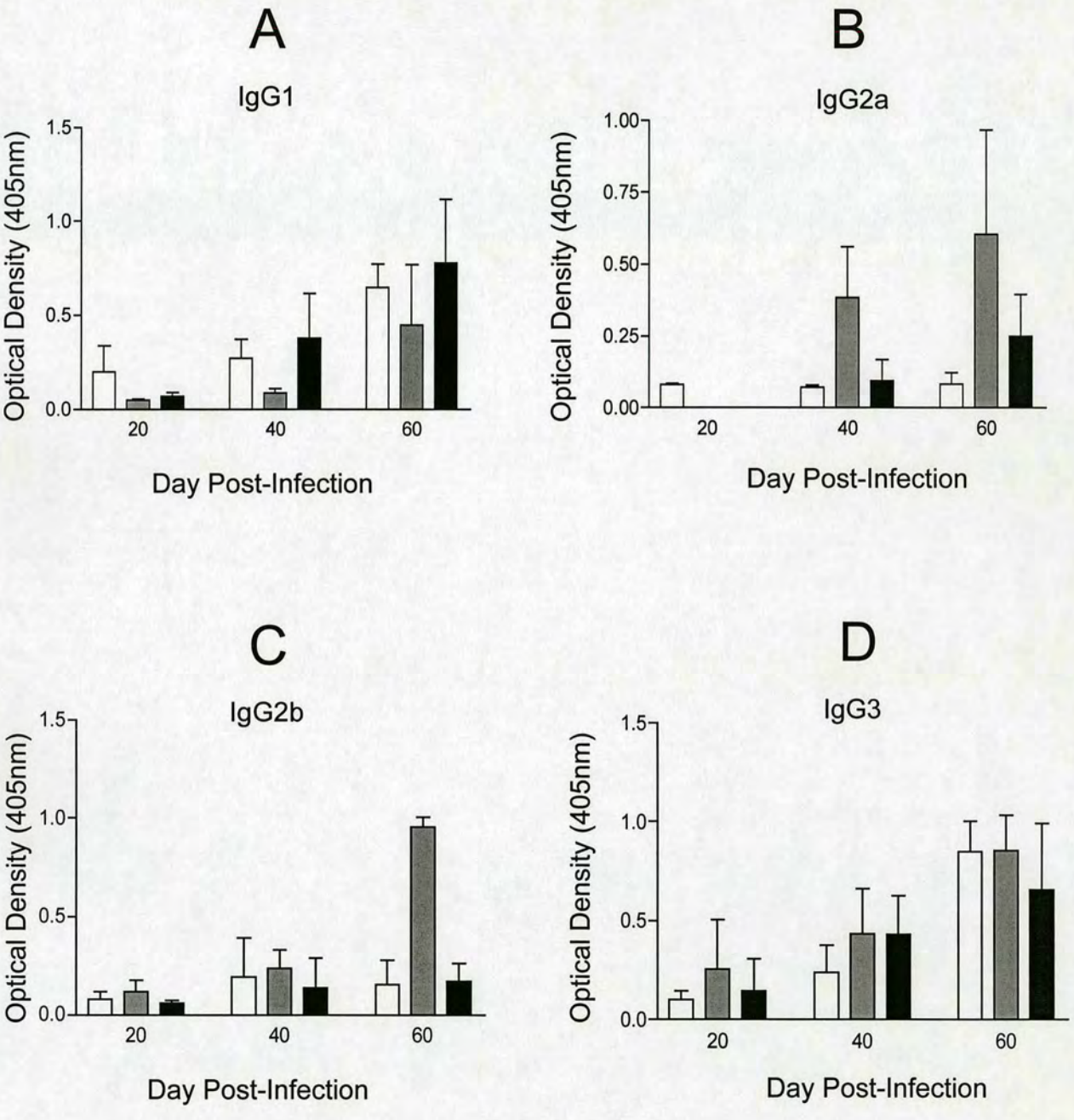


Figure 3:3. Antibody isotype levels in mice infected with *L. sigmodontis* at 20, 40 and 60 days post-infection. *L. sigmodontis* specific IgG1 (A), IgG2a (B), IgG2b (C) and IgG3 (D) are shown. The white bars represent BALB/c mice, the grey bars represent C57BL/6 IL4-/-mice and the black bars represent C57BL/6 mice. The error bars represent the standard deviation between mice in each group.

with BALB/c mice at this time point after infection (Fig. 3:3B). There was little difference in the levels of IgG1 or IgG3 produced against *L. sigmodontis* between the three genotypes of mice at day 60 post-infection (Figs. 3:3A and 3:3D).

3:2:4 Resistance in C57BL/6 mice correlates with the formation of antibody-independent granulomatous cysts around adult filarial nematodes.

Necropsy of resistant C57BL/6 mice infected with *L. sigmodontis* at day 60 post-infection revealed the presence of a number of parasites that were encysted (Fig. 3:4A). In contrast most parasites recovered from BALB/c and C57BL/6 IL4-/- mice appeared healthy (Fig. 3:4B). After sectioning these encysted parasites, Geimsa stain revealed that the material forming the cyst was of a cellular nature (Fig. 3:4C). We hypothesised that one possible mechanism of encystment may involve antibodies. Therefore we decided to ask whether antibodies were necessary for encystment by infecting C57BL/6 mice that have a defect in the μ-chain and are thus deficient in B cells (μMT mice) (Kitamura *et al.*, 1991). μMT mice were equally resistant to wild type C57BL/6 mice (Le Goff *et al.*, 2002). Of the 5 mice per genotype infected, only one mouse in each group was still infected with live *L. sigmodontis* parasites at day 60 post-infection. Additionally we found dead, encysted parasites in the C57BL/6 μMT mice indicating that antibody is not required to form granulomatous cysts around adult *L. sigmodontis* parasites.

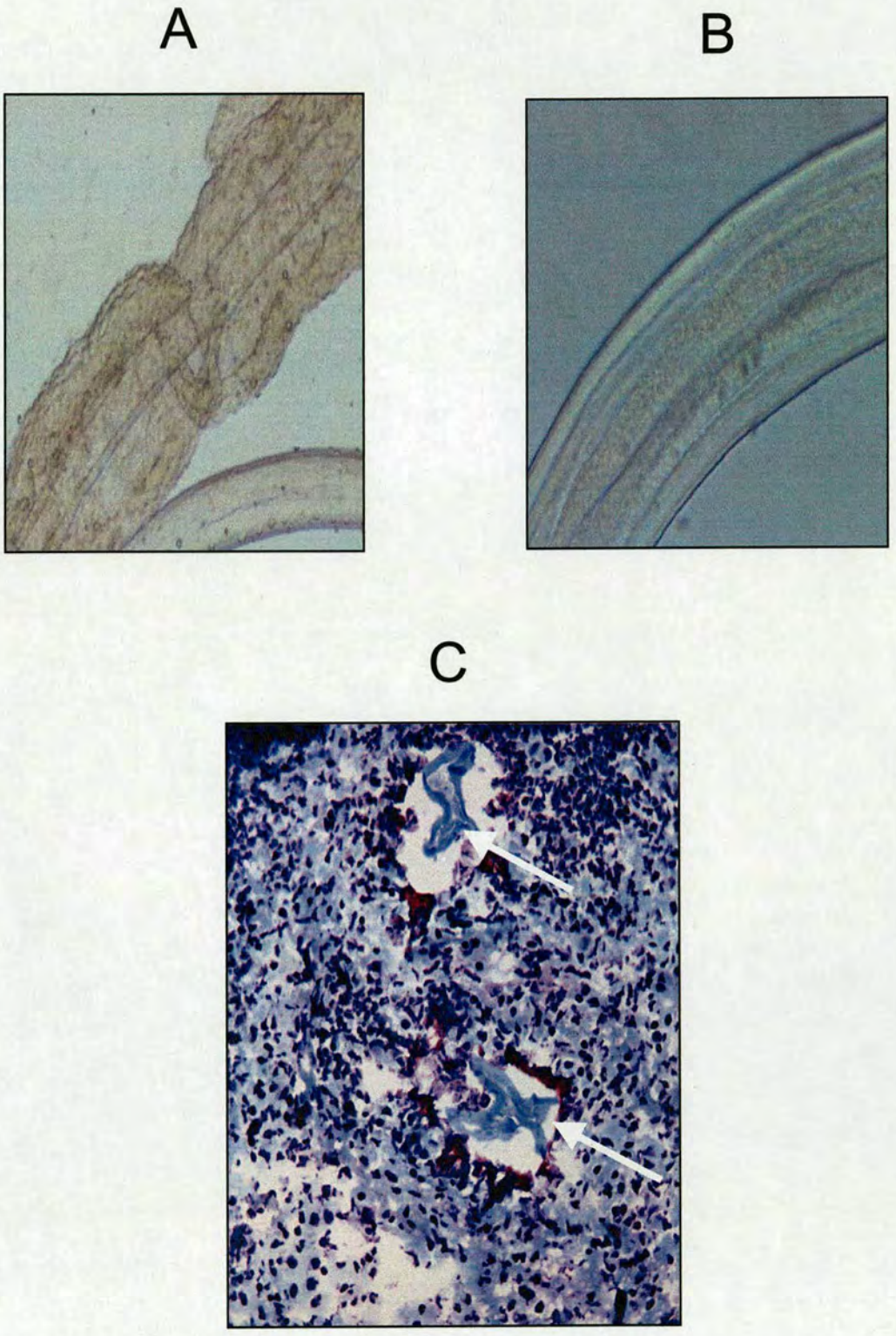


Figure 3:4. Formation of granulomatous nodules around adult parasites in C57BL/6 mice. A nodule from a C57BL/6 mouse is shown in A (X 100). A healthy parasite removed from a C57BL/6 IL4-/- mouse is shown in B (X 100). (C) shows a 5 μm thick section of an encapsulated adult parasite stained in Geimsa (X 200). Nuclei are clearly visible demonstrating the granulomatous nature of the material encapsulating the parasite Arrows show the placement of the parasite within the granulomatous cyst.

Studies in other laboratories have shown that BALB/c mice do not indefinitely harbour *L. sigmodontis* parasites and begin to clear infection from 80 days post-infection onwards (Marechal *et al.*, 1996; Al-Qaoud *et al.*, 2000). Additionally encysted adult nematodes have been recovered from BALB/c mice from this time point (Al-Qaoud *et al.*, 2000) indicating that this mechanism is delayed in BALB/c susceptible mice. In BALB/c mice the granulomatous material has been found to be composed of a layer of neutrophils and neutrophils-derived material immediately adjacent to the nematode cuticle (Al-Qaoud *et al.*, 2000).

We examined the composition of the granulomatous cyst in C57BL/6 mice using fluorescence microscopy by staining sections of encysted nematodes with a fluorescence-labelled antibody specific for mouse neutrophils. We were able to determine that the encystment of adult nematodes in C57BL/6 mice is similar to that in BALB/c mice as the antibody stained a region directly adjacent to the nematode surface (Figs. 3:5A and 3:5B). This staining pattern was not observed in sections stained with an isotype control antibody (Figs. 3:5C and 3:5D).

3:3 Discussion

The role of type 2 responses in filarial nematode infection is yet to be determined. Studies investigating murine gut helminths indicate that in the intestine, a type 2 response is protective (Else *et al.*, 1994; Finkelman *et al.*, 1997; Else and Finkelman, 1998; Finkelman *et al.*, 1999), although the mechanisms by which a type 2 response

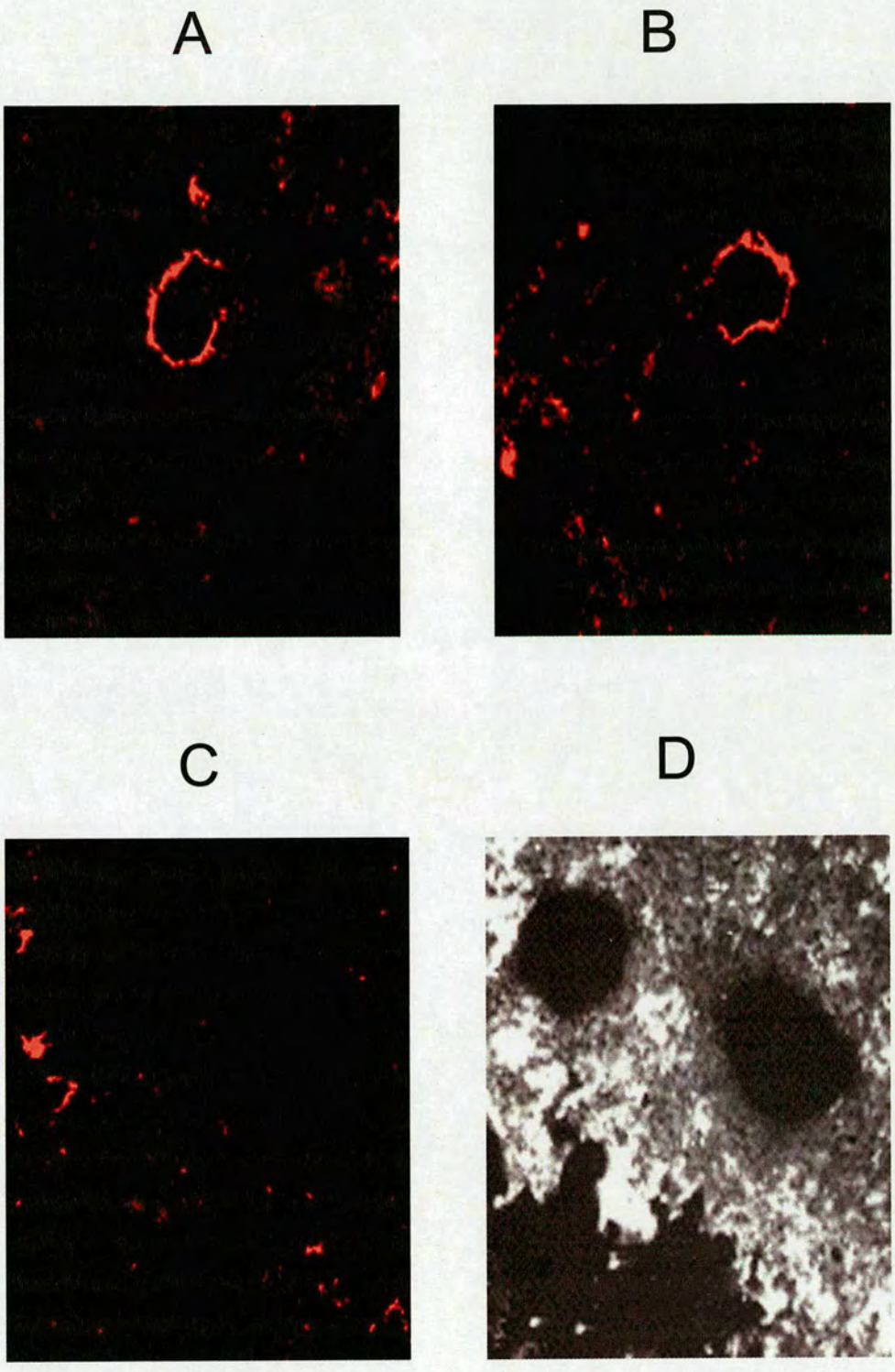


Figure 3:5 Granulomatous nodules around adult parasites have a neutrophilic component. 5 μm thick sections were stained with PE-labelled antibody specific against mouse neutrophils (X 200) (A and B). Sections stained with PE-labelled isotype control antibody are shown in C and D.

protects are parasite specific (Urban *et al.*, 1998; Artis *et al.*, 1999; Urban *et al.*, 2000). Lymphatic filarial nematodes do not reside in the gut, but migrate through, and settle, in the lymphatic vessels. In the case of the murine model *L. sigmodontis* the parasites migrate to the thoracic cavity where they moult to become adults and reproduce (Marechal *et al.*, 1996). BALB/c mice are the only mouse strain that allows the successful completion of the life cycle of *L. sigmodontis*. The immune responses mounted by BALB/c mice and resistant B10.D2 mice to *L. sigmodontis* have previously been compared (Marechal *et al.*, 1997). Both mouse strains mounted type 2 responses against *L. sigmodontis*. However the type 2 response, and in particular the production of IL4, was much greater in the susceptible BALB/c mice. This study validated the use of *L. sigmodontis* in the study of the immunobiology of filarial nematodes but did not come to any firm conclusions regarding the role of type 2 responses in filarial infection.

The finding that IL4 production against adult *L. sigmodontis* parasites in BALB/c mice was significantly higher than in the resistant B10.D2 strain (Marechal *et al.*, 1997) suggested that it may play a role in the susceptibility to infection. However infections of IL4 deficient BALB/c mice indicated that IL4 activated mechanisms contribute to the control of circulating microfilariae rather than playing any role in the establishment of filarial infection (Volkmann *et al.*, 2001). In this study we have extended the work of Marechal *et al.* (1997) and Volkmann *et al.* (2001) to examine the role of type 2 responses, and more specifically the type 2 signature cytokine, IL4, in the resistance of C57BL/6 mice to the establishment of *L. sigmodontis* infection and

the production and circulation of microfilariae. We have found IL4 to be a critical component of the anti-parasite responses in C57BL/6 mice as C57BL/6 IL4-/- mice become as susceptible as BALB/c mice to the establishment of *L. sigmodontis* infection (Fig. 1A). IL4 appears to affect parasite survival between 20 and 40 days post-infection when the L4 to adult moult occurs (Fig. 1A) (Marechal *et al.*, 1996).

In addition to damaging moulting parasites, IL4 effector mechanisms may also prevent the development and / or circulation of Mf. In these studies we have observed that a third of C57BL/6 IL4-/- mice had circulating Mf, again similar to BALB/c susceptible mice (Fig. 3:1B). It is unclear whether these mice became microfilaraemic because more parasites were able to survive to parenthood, or whether IL4 reduces fecundity of adult parasites or plays a role in the clearance of microfilariae from the blood stream. Studies with *L. sigmodontis* (Volkmann *et al.*, 2003) and the feline filarial parasite *Brugia pahangi* (Devaney *et al.*, 2002) indicate that IL4 may target the fecundity of adult parasites in BALB/c mice. However the clearance of Mf from the blood of BALB/c IL4-/- mice has been shown to be equivalent to wild type BALB/c mice (Volkmann *et al.*, 2003). Thus extrapolation of data from these studies with the BALB/c mouse strain indicate that C57BL/6 IL4-/- mice may become microfilaraemic because of both the increased number of surviving adult nematodes (Fig. 3:1A) and possibly also the increased fecundity of these adults, but not because of an impaired clearance of microfilariae.

BALB/c mice tend to be more susceptible than C57BL/6 mice to *L. sigmodontis* infection, even in the early time points post-infection (Fig. 3:1A). This apparent difference did not reach statistical significance although the same trend has been observed in other studies (Marechal *et al.*, 1996; Hoffman *et al.*, 2000). BALB/c mice also appeared to be more susceptible at day 20 post-infection than C57BL/6 IL4-/- mice indicating that there is a limited role for IL4 in mediating resistance immediately after infection.

IL4 could mediate resistance to *L. sigmodontis* in C57BL/6 mice in a variety of ways. Initially it could down-regulate components of type 1 inflammatory responses that have been hypothesised to be required for the development of filarial nematodes (Ravindran 2001). Secondly it could enhance effector mechanisms generated from type 2 responses, such as the generation of IgE and IgG1 antibodies from B cells (Snapper and Paul 1987b; Snapper *et al.*, 1988b; Purkerson and Isakson 1992), the degranulation of mast cells by IgE cross-linking (Chen and Enerback 2000) and the migration and/or activation of granulocytes such as eosinophils, basophils and neutrophils that have all been implicated in mediating the death of nematodes (Al-Qaoud *et al.*, 2000; Martin *et al.*, 2000b; Falcone *et al.*, 2001; Saeftel *et al.*, 2001).

Indeed, in these studies we have demonstrated that IL4 is a mediator of granulomatous encystment of adult parasites in the thoracic cavity. It is unknown whether the observed encystment is a consequence of adult death, and acts to clear dead parasites from the cavity, or whether encystment is a mechanism that actively

kills adult *L. sigmodontis*. The observation that parasites can be partially encysted and moving indicates that the latter may be true (Fig. 3:4A). We observed few cysts in infections of BALB/c mice or C57BL/6 IL4-/- mice at day 60 post-infection indicating that this may be a mechanism by which IL4 provides protection against *L. sigmodontis* in C57BL/6 mice. The encystment of parasites was not dependent on IL4 mediated antibody isotype switching as encysted parasites were found in C57BL/6 μMT mice that are deficient in B cells (Le Goff *et al.*, unpublished observation). Additionally C57BL/6 μMT mice were equally resistant as wild type C57BL/6 mice (Le Goff *et al.*, 2002).

Although we did not observe many encysted adult parasites in BALB/c mice, other studies that have examined day 80 post-infection have demonstrated that parasite encystment occurs later in this susceptible strain of mice (Al-Qaoud *et al.*, 2000; Saeftel *et al.*, 2001). Thus the infection phenotype of BALB/c mice could be described as delayed resistant phenotype rather than susceptible. It is unknown whether this is also the case for C57BL/6 IL4-/- mice. The observed encystment was found to consist of a layer of neutrophils directly adjacent to the nematode cuticle (Al-Qaoud *et al.*, 2000). Studies in BALB/c mice deficient in interleukin-5 (IL5) suggest that this cytokine is essential for encystment and late clearance of established adult parasites (Martin *et al.*, 2000a; Al-Qaoud *et al.*, 2000; Volkmann *et al.*, 2003). However IL5 is not essential for the innate resistance of C57BL/6 mice (Le Goff *et al.*, 2000b) and encysted parasites can be found in the thoracic cavity at 60 days post-infection (Le

Goff *et al.*, unpublished observations). This indicated that encystment of adult parasites in the C57BL/6 mouse strain may be different to that in BALB/c mouse strain. However we have examined the composition of encysted parasites taken from C57BL/6 wild type mice and were able to determine that the cysts observed in C57BL/6 mice do have some similarities with those observed in BALB/c mice (Al-Qaoud *et al.*, 2000) (Figs. 3:5A and 3:5B).

IL4 is an integral part of the immune response against filarial nematodes. Upon examination of the immune response at day 60 post-infection, when the parasite burden is statistically different between BALB/c susceptible mice and C57BL/6 resistant mice, we have seen a similar production of IL4 by splenocytes in response to adult *L. sigmodontis* (Fig. 3:2A). IL10 has been associated with susceptibility to murine (Osborne and Devaney, 1999) and human (Doetze *et al.*, 2000) filarial infection. Indeed we have shown in these experiments that parasite-specific IL10 is secreted from the splenocytes of BALB/c susceptible mice but not C57BL/6 resistant mice (Fig 3:2C). Therefore it could be argued that IL10 alone may be mediating susceptibility in these experiments, perhaps through T regulatory or Th3 cells (Doetze *et al.*, 2000). However C57BL/6 IL4-/- mice secrete less IL10 than BALB/c mice, yet harbour a similar number of parasites. It is possible that the relative amounts of IL4 and IL10 may determine resistance or susceptibility to *L. sigmodontis* infection.

We have shown in these studies that IL4 protects C57BL/6 mice against *L. sigmodontis* (Fig. 3:1A) and wild type mice of this strain do secrete parasite-specific IL4 (Fig. 3:2A), but not IL10 (Fig. 3:2C). On the other hand BALB/c mice secrete both parasite-specific IL4 and IL10 (Fig 3:2A and 3:2C). It is possible that IL4 can only protect mice against *L. sigmodontis* infection in the absence of IL10. IL10 may be able to down-regulate the activation and/or migration of IL4-induced effector cells, such as the neutrophils that form an integral component of the granulomatous cyst around adult *L. sigmodontis* parasites (Figs. 3:5A and 3:5B; Al Qaoud *et al.*, 2000; Saeftel *et al.*, 2001). The activation of neutrophils can be promoted by IL4 (Boey *et al.*, 1989). However some aspects of neutrophil action, such as the secretion of proinflammatory cytokines (Cassatella *et al.*, 1993) and chemokines (Kasma *et al.*, 1994), are inhibited by IL10 (see Cassatella 1998). Therefore BALB/c mice and C57BL/6 IL4-/- mice may have similar recovery rates, despite secreting different amounts of parasite-specific IL10, because IL10 can counter-regulate the anti-parasite effector mechanisms that are triggered by the IL4 that is simultaneously secreted in BALB/c mice, but not C57BL/6 IL4-/- mice. This theory could be tested by infecting BALB/c mice that are deficient in IL10.

BALB/c mice, but not C57BL/6 IL4-/- mice or C57BL/6 wild type mice, mounted a measurable antibody response at day 20 post-infection consisting of IgG1 and IgG2a isotypes (Fig. 3:3A and 3:3B). C57BL/6 IL4-/- and C57BL/6 wild type mice had nematode recovery rates that were similar, and less than, that in BALB/c mice at this

time point. Although this early antibody response correlates with the susceptibility of BALB/c mice early after infection in this study, this data is contrary to that found by Marechal *et al.*, (1997) who observed anti-parasite IgG1 and IgG2a at day 20 post-infection in resistant B10.D2 but not susceptible BALB/c mice. Therefore the significance of early immune responses to resistance and susceptibility to *L. sigmodontis* infection is unclear.

The presence of live adult nematodes was associated with secretion of parasite-specific IFNγ at day 60 post-infection (Fig. 3:2B). It is not clear whether this response was a cause, or a consequence, of the presence of live adult *L. sigmodontis* at this time point in these mice (Fig 3:1A). Although susceptibility to the establishment of infection in these experiments correlates with the presence of IFNγ at day 60 post-infection, it also correlates with the presence of circulating microfilariae (Fig. 3:1B). Microfilariae have been shown to be inducers of type 1 responses in the *B. malayi* system (Lawrence *et al.*, 1994). On the other hand infection of BALB/c mice deficient in IFNγ (IFNγ -/-) indicate that this response in necessary for parasite clearance (Saeftel *et al.*, 2002). Therefore BALB/c mice and C57BL/6 IL4-/- mice could be susceptible due to a delay in mounting a type 1 response, and the observed IFNγ secretion could be the beginning of a response that will clear these parasites from the cavity. Although no IFNγ was secreted from splenocytes of C57BL/6 mice in response to adult *L. sigmodontis* at day 60 post-infection (Fig. 3:2B), anti-parasite IgG2a, produced by B cells in response to IFNγ (Snapper and Paul, 1987a), was measured at

this time point post-infection in these mice (Fig. 3:3B). This indicates that a type 1 response may have occurred at a time point in these mice prior to day 60 post-infection. However it is also possible that no splenocyte IFNγ was measured in the spleens from these mice in response to the parasite because the IFNγ driving the production of this isotype was produced in the local draining lymph nodes rather than the spleen.

An interesting feature of the *L. sigmodontis* murine filarial infection is that the susceptibility and resistance pattern in C57BL/6 and BALB/c genetic lines of mice is identical to the protozoan parasite *Leishmania major* (Sacks and Noben-Trauth, 2002) but the opposite to the gut helminth *T. muris* (Faulkner *et al.*, 1998). *L. major* initially induce a type 2 response (Scott *et al.*, 1996) but C57BL/6 mice are resistant as they are subsequently able to mount a strong type 1 response (Wang *et al.*, 1994a). Conversely C57BL/6 mice mount type 1 responses to *T. muris* and are susceptible as they fail to mount protective type 2 responses (Grencis 2001). Therefore the resistance of C57BL/6 mice is a common feature of *L. sigmodontis* and *L. major* infections, although the effector mechanisms against these two parasites are likely to be very different.

In conclusion this study has shown that IL4 is necessary for resistance to infection with *L. sigmodontis* in C57BL/6 mice. IL4 appears to act at several different time points during infection. We have evidence that IL4 can mediate parasite damage

during the moult from L4 to adult, abrogate the circulation of microfilariae and also facilitate the formation of parasite-damaging granulomatous cysts.

CHAPTER 4

The Source of IL4 Providing Protection in C57BL/6 Mice

4:1 Introduction

Filarial nematodes will evoke immune responses from both the innate and adaptive arms of the host immune system. We have previously shown that resistance of C57BL/6 mice to establishment of infection with the rodent filarial nematode *Litomosoides sigmodontis* is dependent on the type 2 cytokine IL4 (Le Goff *et al.*, 2002). As well as forming a hallmark of adaptive immune responses mounted by the host against filarial nematodes (Ottesen 1992; Lawrence and Devaney 2001), this cytokine is evoked from the innate immune response by murine filarial nematodes early after infection (Osborne and Devaney 1998; Balmer and Devaney 2002). Therefore the IL4 produced by C57BL/6 mice that provides resistance against the establishment of infection with *L. sigmodontis* could originate from the innate immune response, the adaptive immune response, or both.

We hypothesised that the adaptive immune response is likely to be an important source of protective IL4 because previous studies suggest that the adaptive immune response plays a protective role in defending the host against filarial infection. SCID and RAG -/- mice both have mutations that prevent them from generating functional B and T cells and are susceptible to the development of *Brugia malayi* infection to patency (Nelson *et al.*, 1991; Babu *et al.*, 1999). This suggests that B and T cells,

which are both capable of producing IL4, are essential for resistance to filarial nematodes. Athymic mice, lacking only T cells, are susceptible to infection with *B. pahangi* (Suswillo *et al.*, 1980; Vincent *et al.*, 1982; Paciorkowski *et al.*, 2000) although individual roles for CD4+ T cells alone (Rajan *et al.*, 1994) or CD8+ T cells alone (Rajan *et al.*, 1992) in mediating protection to *Brugia* infection have so far not been demonstrated. Reports on the relative importance of B cells in resistance to *B. malayi* infection are conflicting (Rajan *et al.*, 1995; Babu *et al.*, 1999; Paciokowski *et al.*, 2000) but may be dependent on mouse strain (Babu *et al.*, 1999). Although no studies of *L. sigmodontis* infection in SCID or RAG -/- mice have been reported, it has been demonstrated that CD4+ T helper cells (Al-Qaoud *et al.*, 1997), but not B cells (Martin *et al.*, 2001; Le Goff *et al.*, 2002) protect the host against the establishment of infection with *L. sigmodontis*.

The aim of this study was to investigate the relative importance of IL4 production from each arm of the immune response in the defence of C57BL/6 mice against *L. sigmodontis* infection. Our approach in this investigation made use of mice deficient in the recombination-activating gene 2 (RAG2 -/-). These mice do not produce mature T or B lymphocytes, as they are unable to rearrange their immunoglobulin and T cell receptor loci (Shinkai *et al.*, 1992). However few other effects of this mutation on the immune system were observed in these mice. By reconstituting these mice with splenocytes from mice deficient in IL4 (Kuhn *et al.*, 1991) we were able to create chimeric mice that were able to produce IL4 from the innate arm of the immune response only (Table 4:1). We also generated mice deficient in both RAG 2

CHIMERIC MOUSE	INNATE IMMUNE RESPONSE	ACQUIRED IMMUNE RESPONSE
RAG2 -/- + WT Splenocytes	**IL4**	**IL4**
RAG2 -/- + IL4-/- Splenocytes	**IL4**	
RAG2 / IL4 -/- + WT Splenocytes		**IL4**
RAG2 / IL4 -/- + IL4-/- Splenocytes		

Table 4:1. Creation of chimeric animals that can only produce IL4 from the innate or adaptive immune system.Reconstitution of RAG2 -/- mice and RAG2 / IL4 -/- mice with splenocytes from wild type or IL4 -/- mice provides a model for which to examine the source IL4 that protects C57BL/6 mice against the establishment of infection with *L. sigmodontis*.

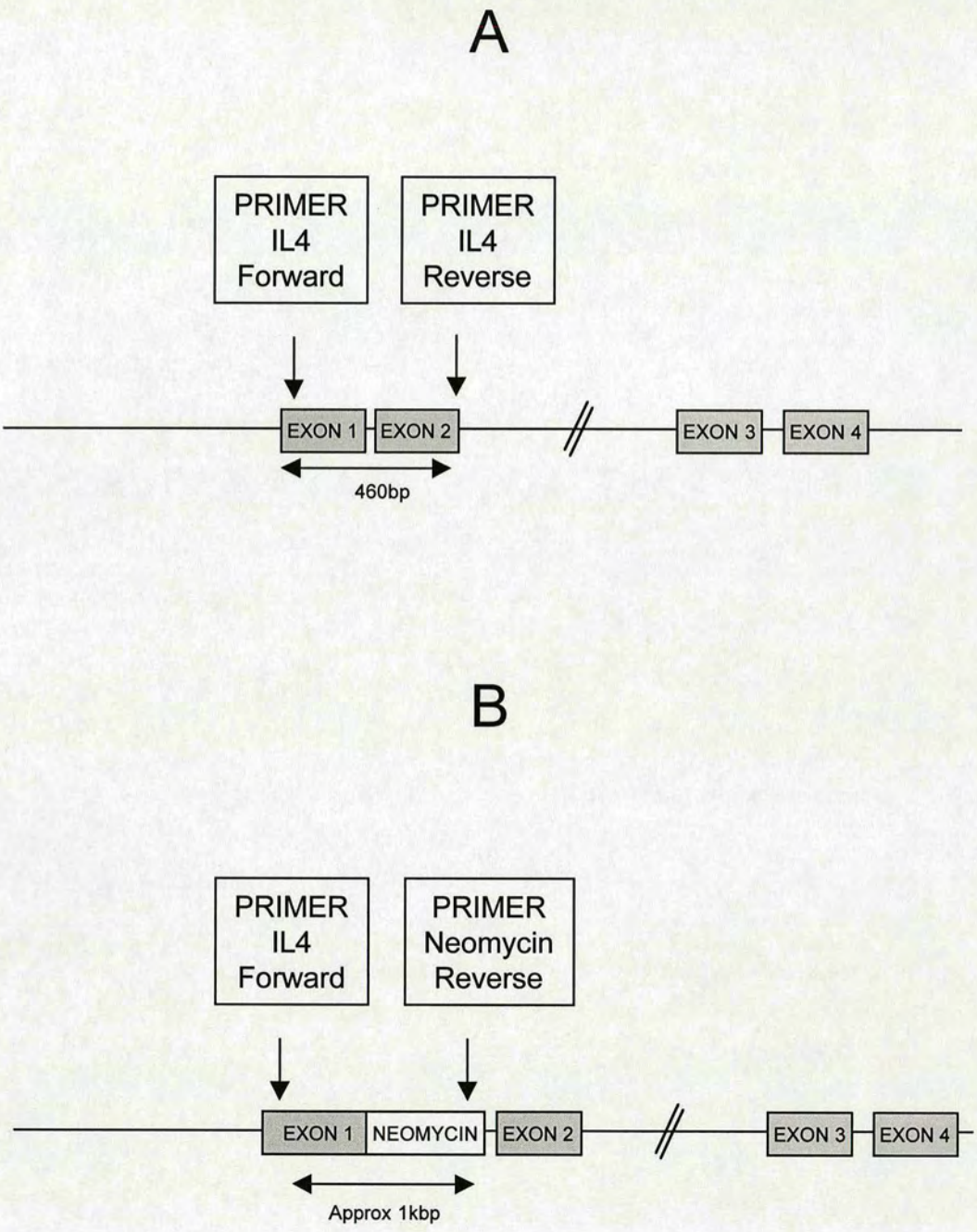


Figure 4:1. Schematic structure of the wild type (A) and knockout (B) IL4 gene (Kuhn *et al.*, 1991) indicating the sites of primer binding in the PCR's used in this study. The diagram is not drawn to any scale.

and IL4 (RAG2 / IL4 -/-) and reconstituted these mice with splenocytes from wild type C57BL/6 mice to create chimeric mice that were able to produce IL4 from the adaptive immune response only (Table 4:1).

By infecting these chimeric mice with *L. sigmodontis*, and examining the outcome of infection 60 days later, we were able to assess the contribution of IL4 from the innate or adaptive immune systems to the resistance of C57BL/6 mice. These studies indicate that IL4 must be present in both arms of the immune response to prevent the establishment of infection with *L. sigmodontis*.

4:2 Results

4:2:1 Generation of RAG2 / IL4 -/- C57BL/6 mice.

Female C57BL/6 RAG2 -/- mice (a kind gift of Prof D. Gray) were crossed with male C57BL/6 IL4 -/- mice (Kuhn *et al.*, 1991) (purchased from Bantam and Kingman) to form mice heterozygote for both genes. The F1 generation from this cross were mated together and the F2 progeny were screened for mice homozygous for both genes. Since mice homozygote for RAG2 deficiency have no mature T or B cells, the peripheral blood was examined for the lymphocytes expressing the B cell co-receptor molecule CD19, and the T cell receptor accessory molecule CD3, by FACS analysis. The number of RAG2 -/- mice obtained conformed to the expectations of Mendelian genetics.

The mice determined to be RAG2-/- mice were screened for IL4 status by PCR of genomic DNA obtained from digesting tail snips. The IL4 -/- mice created by Kuhn *et al.* (1991) were rendered deficient by the introduction of a neomycin gene inserted into the IL4 gene at exon 1 (Fig. 4:1). After determining the presence of genomic DNA in each sample by amplifying the housekeeping gene β-actin (Table 2:1), we used two reactions to determine the IL4 status of the F1 mice. Using the primers IL4-forward and neomycin- reverse (Table 2:1 and Fig. 4:1B) we set up a reaction to amplify the neomycin insert. This reaction should give a product of 350bp for any mouse that contains a transgenic IL4 gene (Bantam and Kingman, personal communication). To differentiate the homozygosity and heterozygosity of mice positive for the neomycin insert, we set up a second reaction to identify the intact IL4 gene. Primers were designed to bind to the beginning of exon 1 for the IL4 gene and to the end of exon 3 (Table 2:1 and Fig. 4:1A). This reaction amplified a band of approximately 400bp in the wild type gene and a band of approximately 1kbp in the transgenic gene.

Mice deemed to be homozygote RAG2-/-, and also homozygote for the IL4 gene containing the neomycin insert, were mated with each other and the resulting F3 generation bred and maintained in filter top cages in animal breeding facilities at the University of Edinburgh.

Randomly chosen mice from the colonies to be used in the experiments for this study were bled from the tail and the blood was examined for the presence of cells with

CD19 or CD3 on their surface. Fig. 4:2 shows the FACS plots representative of the results obtained from this random screen. Circulating B cells and T cells can be seen in the blood of C57BL/6 wild type and IL4-/- mice only (Fig. 4:2A and 4:2B respectively) but not C57BL/6 RAG2-/- or RAG2 / IL4-/- mice (F3 generation) (Fig. 4:2C and 4:2D respectively) confirming the RAG2 status of these mice. Tail digests were also performed on randomly chosen mice from these colonies. Fig. 4:2E shows the PCR results obtained from a mouse representative of each colony for all the tail digests performed. It can be seen that C57BL/6 wild type and RAG2-/- mice are homozygous for the wild type IL4 gene, whilst IL4-/- mice were positive for the neomycin PCR and produced only a high molecular weight band during a PCR to amplify the IL4 gene (Fig. 4:2E). A heterozygote F1 mouse from a mating between IL4-/- and RAG2-/- mice is shown for comparison and, as expected, is positive for the neomycin insert, as well as the wild type IL4 gene (Fig. 4:2E). Finally, the mouse from the colony deemed to be RAG2 / IL4 -/- show the same phenotype as the IL4-/- mice demonstrating that this mouse only had IL4 genes containing the neomycin insert.

4:2:2 RAG2-/- mice and IL4-/- mice are equally susceptible to the establishment of L. sigmodontis *infection.*

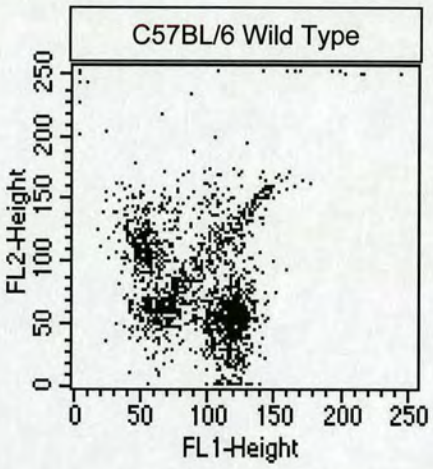
Before reconstituting any mice, we wanted to determine the infection phenotype of RAG2-/- mice and RAG2 / IL4-/- mice. Three experiments were carried out comparing the adult nematode recovery rate from RAG2-/- mice, IL4-/- mice and wild type C57BL/6 mice at 60 days post-infection. The number of adult nematodes

recovered from each mouse was square-root transformed to allow meta analysis of three different experiments. No interaction between experiment and treatment was detected in the meta- analysis (ANOVA, $P>0.05$). The recovery rate from RAG2 -/- mice was greater than that from C57BL/6 wild type mice (Tukey's Pairwise Comparisons test, $P<0.05$), but was not different from the IL4-/- mice (Tukey's Pairwise Comparisons test, $P>0.05$) (Fig. 4:3A). No difference was observed in the recovery rate of adult filarial nematode at 60 days post-infection between RAG2 / IL4 -/-, RAG2 -/- and IL4-/- mice (Kruskal Wallis, $P>0.05$) (Fig. 4:3C). Around 10% of the original L3 innoculum were recovered from the thoracic cavity of RAG2-/- mice in all these experiments.

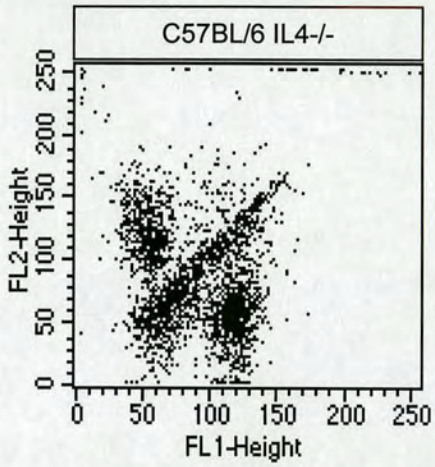
We also examined the % of mice infected that developed patent infection in these initial experiments. As expected no circulating microfilariae (Mf) could be detected in C57BL/6 wild type mice (Fig. 4:3B). Around 10% of infected IL4-/- mice became patent in experiments comparing mice deficient in only one gene (Fig. 4:3B). This level is lower than expected from previous studies in our laboratory (Le Goff *et al.*, 2002). In the experiment comparing doubly deficient mice with mice deficient in only one gene, no circulating Mf could be detected in any of the IL4-/- mice (Fig. 4:3D). Circulating Mf were detected in some of the RAG2-/- and RAG2 / IL4 -/- mice demonstrating that these animals can develop microfilariaemia. Similarly to the susceptible BALB/c strain (Petit *et al.*, 1992) not more than 50% of these animals had circulating Mf.

Figure 4:2. The typing of RAG2 / IL4-/- mice. Tail blood was typed for Circulating lymphocytes by FACS analysis.The dead cells were gated out for each analysis. Peripheral cells were stained using antibodies to CD19 conjugated to FITC, shown on the x-axis (FL1), and to CD3 conjugated to PE and shown on the y-axis (FL2). The profile of a mouse representative of those from the following colonies are shown : C57BL/6 wild type (A), C57BL/6 IL4 -/- (B),C57BL/6 RAG2-/- (C) and RAG2 / IL4-/- (D). PCR reactions for beta-actin (BA , 600bp), neomycin insert (neo, 350bp) and IL4 (400bp or 1kbp for wild type and transgenic genes respectively) were carried out to determine the IL4 status of the colonies of mice used in these experiments (E).

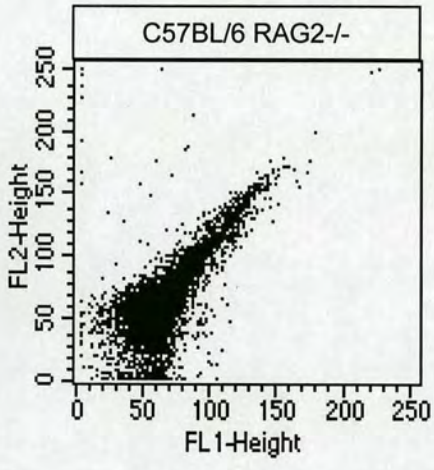
A


C57BL/6 Wild Type
FL2-Height
FL1-Height
0
50
100
150
200
250

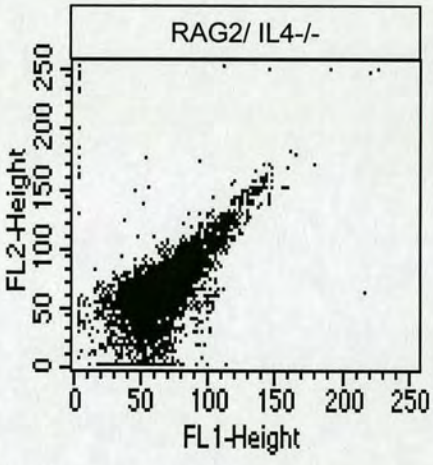
B


C57BL/6 IL4-/-
FL2-Height
FL1-Height
0
50
100
150
200
250

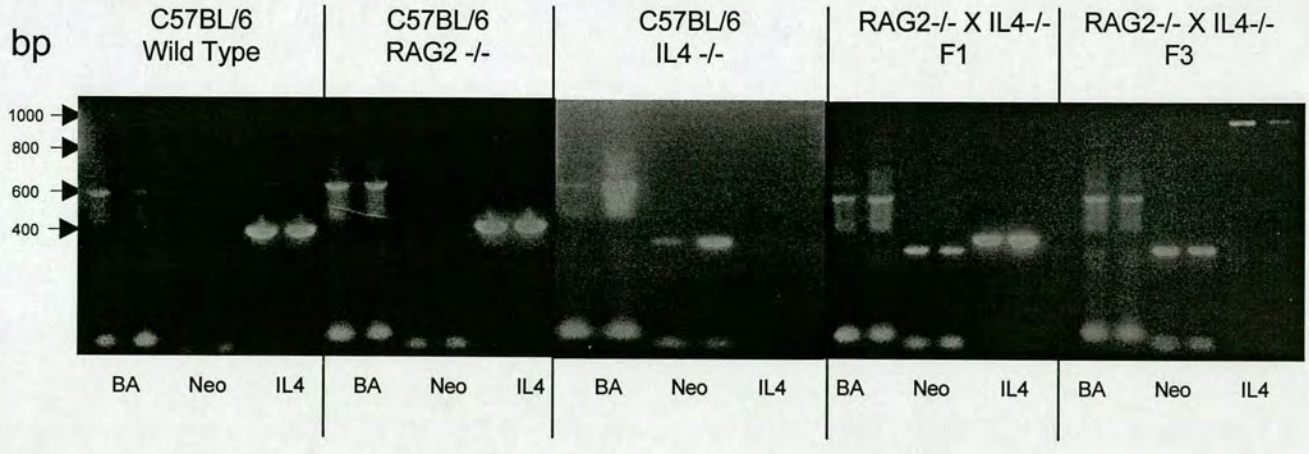
C


C57BL/6 RAG2-/-
FL2-Height
FL1-Height
0
50
100
150
200
250

D


RAG2/ IL4-/-
FL2-Height
FL1-Height
0
50
100
150
200
250

E


bp
C57BL/6 Wild Type
C57BL/6 RAG2 -/-
C57BL/6 IL4 -/-
RAG2-/- X IL4-/- F1
RAG2-/- X IL4-/- F3
1000
800
600
400
BA
Neo
IL4

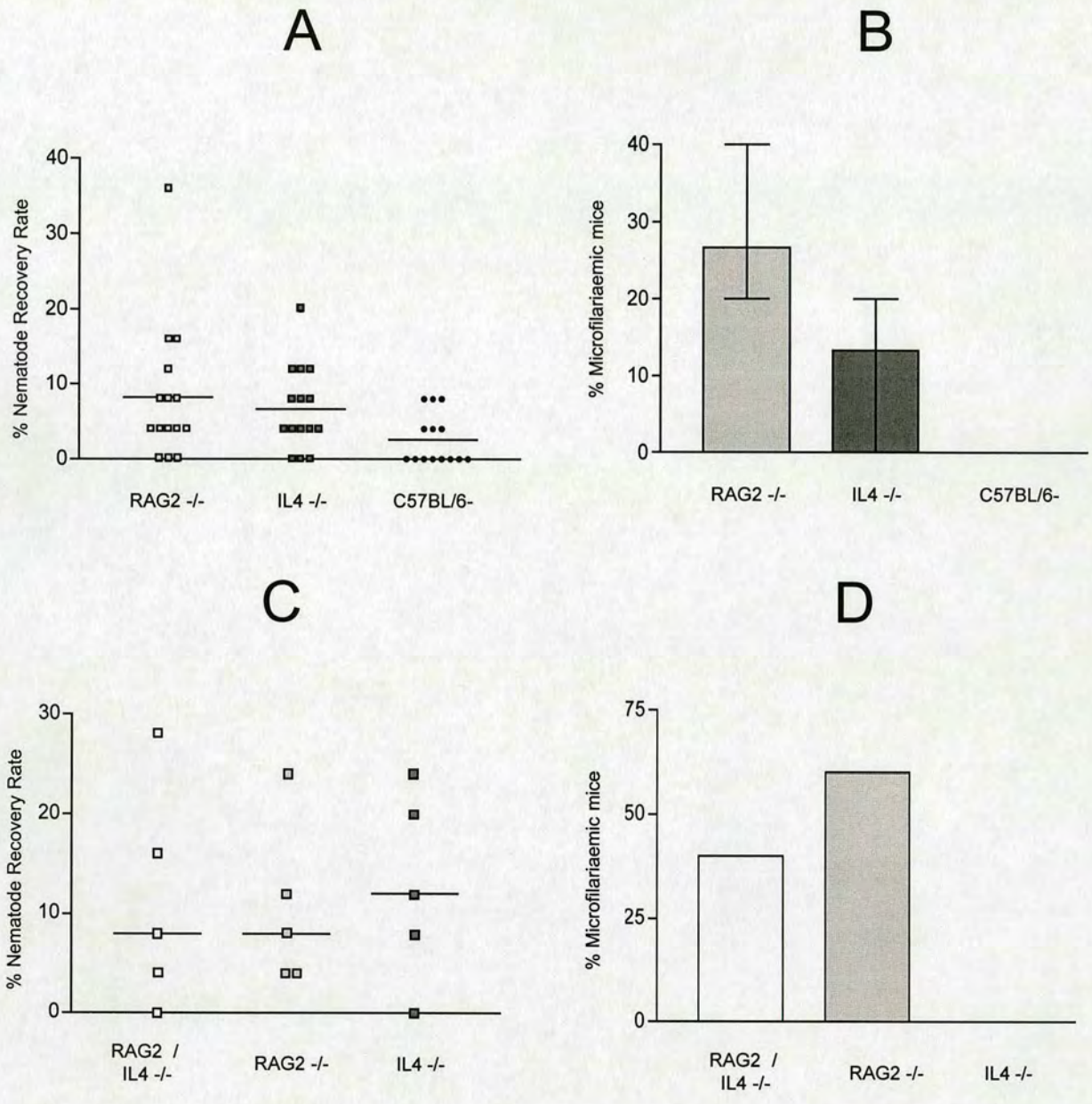


Figure 4:3. Nematode recovery rates and the % of mice with circulating Microfilaria in RAG2 -/- and IL4 / RAG2 double knockout animals. RAG2-/- mice are compared with IL4-/-and wild type C57BL/6 mice in A and B. The results of three separate experiments are shown in A and B. RAG2 / IL4 -/- animals are compared with mice singly deficient in RAG2 or IL4 in C and D. Each square denotes the recovery rate for one infected mouse and the bars represent the mean (A) or the median (C). The error bars in B represent the maximum and minimum % of microfilariaemic mice observed amongst the three experiments represented.

4:2:3 The protocol used to reconstitute RAG2-/- and RAG2 / IL4-/- mice was successful.

The mice to be used in each experiment were sub-lethally irradiated on day 0 of each experiment and reconstituted with 1 x 10^8 splenocytes intravenously the following day. Mice were infected with *L. sigmodontis* on day 4. To assess the efficacy of this protocol we examined the phenotype of the splenocytes of these mice at the end of each experiment. The results from one experiment is shown in Fig. 4:4. This protocol was successful in reconstituting mice, as the levels of CD4+ T cells (Fig. 4:4A), CD8+ T cells (Fig. 4:4B) and CD19+ B cells (Fig. 4:4C) were comparable to wild type C57BL/6 mice in all cases. The reconstituted cells were functional as the T cells were able to respond to stimulation with concanavalin A (Fig. 4:5A) and the B cells were able to produce parasite specific IgG antibodies (Fig. 4:5B).

4:2:4 IL4 production from the adaptive immune response is required for protection against the establishment of L. sigmodontis *infection.*

RAG2-/- mice were reconstituted with cells from wild type C57BL/6 mice or IL4-/- mice and the number of surviving adult nematodes enumerated at 60 days post-infection. Three experiments were performed and were meta-analysed after square root transformation of the number of nematodes recovered from each mouse. IL4 -/- mice were only included in two of these experiments as an additional control for susceptibility and were not included in the analysis. The recovery rate was different between the groups of mice tested in this analysis (ANOVA $P<0.01$) (Fig. 4:6A). This difference arose from the C57BL/6 wild type mice which had significantly

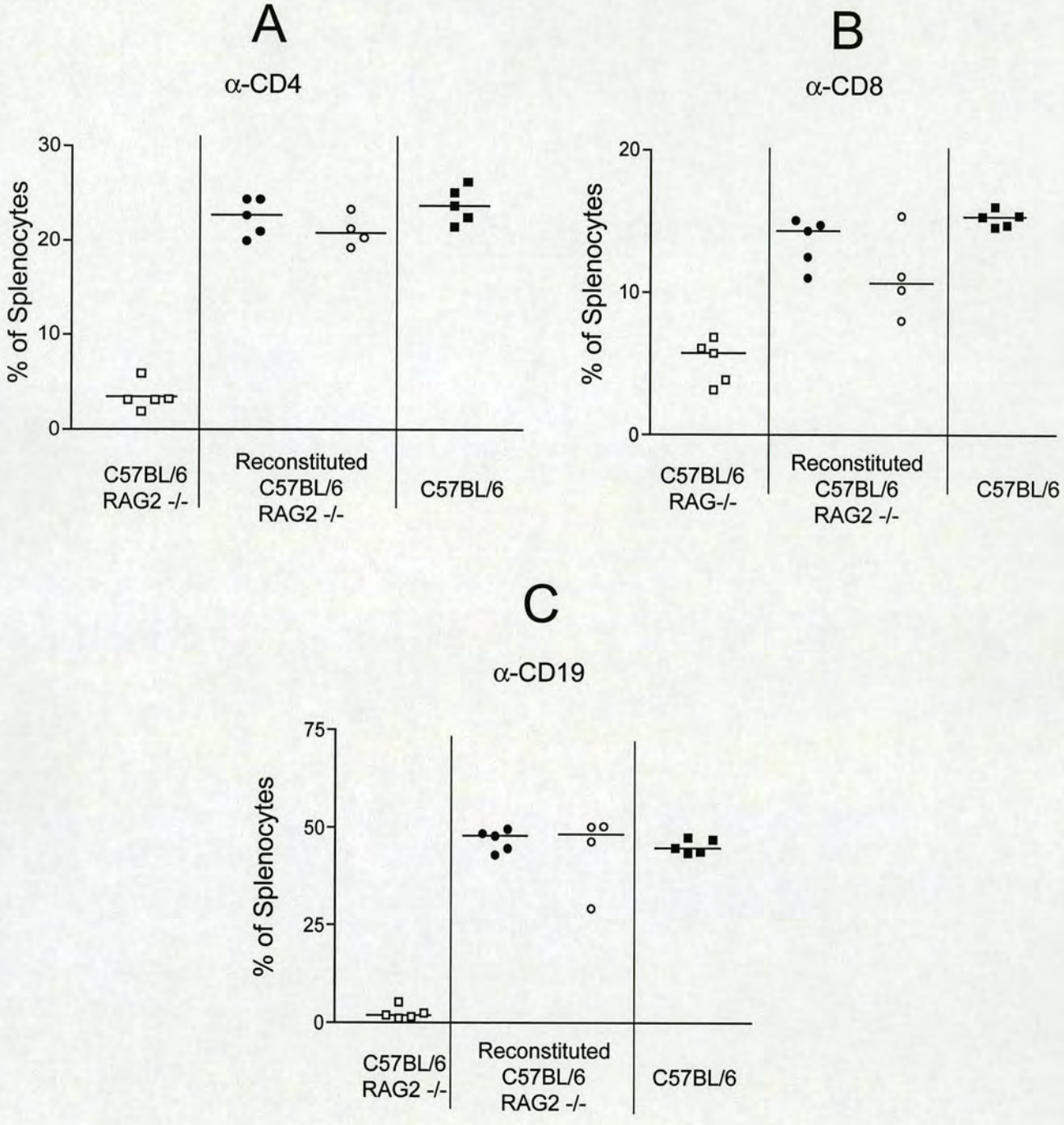


Figure 4:4. Splenocyte compositions of reconstituted C57BL/6 RAG2 -/- mice at 60 days post - infection with *L. sigmodontis* from one representative experiment. Whole splenocytes were stained with α-CD4-PE (A), α-CD8-Cychrome (B) and α-CD19-FITC (C). The dead cells were gated out of the analysis. In each graph the white squares represent the RAG -/- mice and the black squares represent C57BL/6 wild type mice. The black circles represent RAG -/- mice that were reconstituted with 1 x 10^8 splenocytes from C57BL/6 wild type mice and the white circles represent RAG-/- mice that were reconstituted with 1 x 10^8 splenocytes from C57BL/6 IL4 -/- mice. Each symbol represents one animal. Each bar represents the median value for each group.

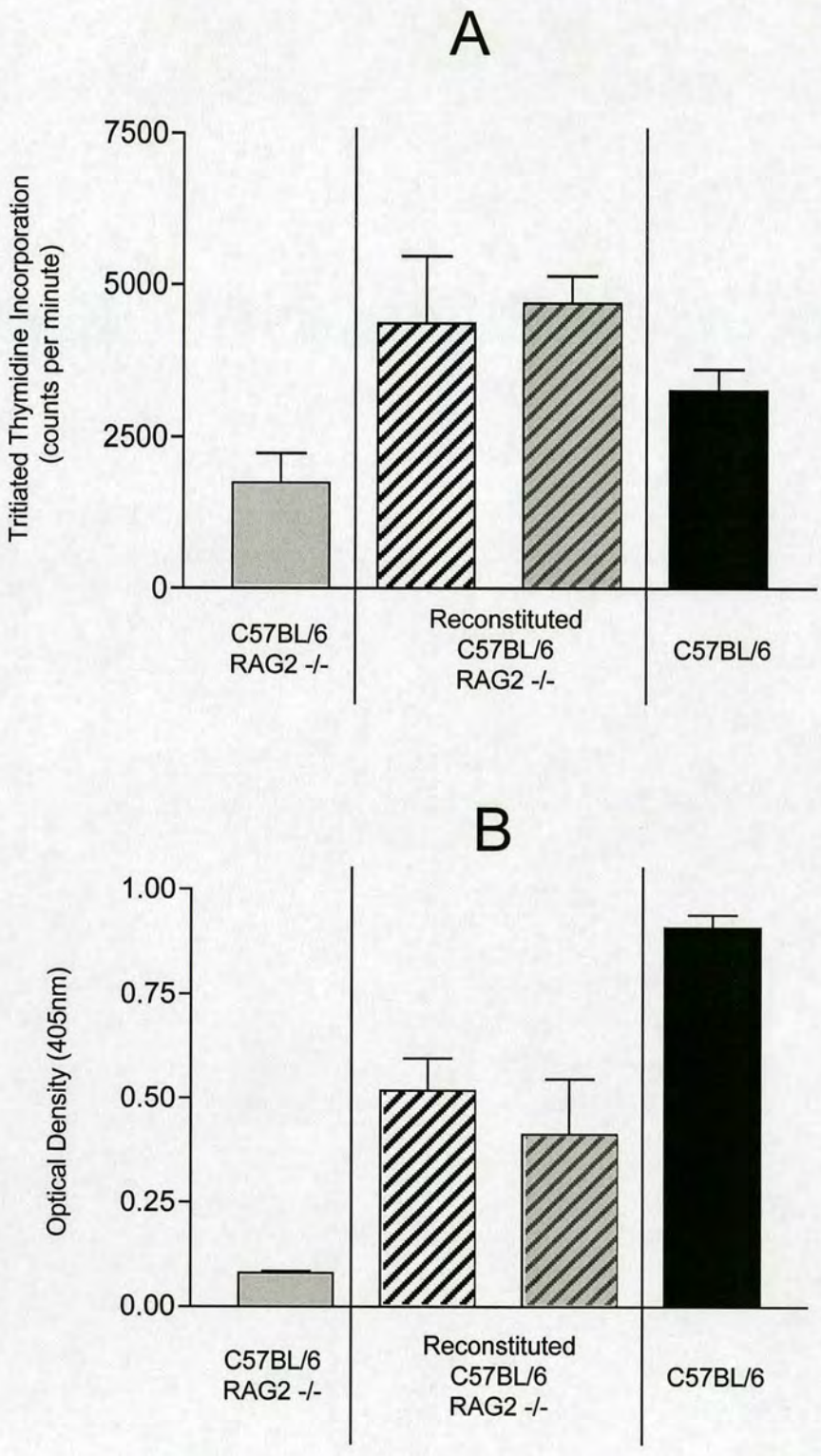


Figure 4:5. Reconstituted cells are still present and functional by the end of 60 days of infection with *L. sigmodontis*. Splenocytes from reconstituted mice were stimulated with the T cell mitogen Con A and the thymidine incorporation examined as a measure of proliferation (A). Circulating total IgG antibody against *L. sigmodontis* was measured by ELISA to check the viability of the reconstituted splenic B cells (B).

lower parasite recovery rates than the RAG2-/- or RAG2-/- mice reconstituted with IL4-/- splenocytes (Tukey's Multiple Comparison test, both $P<0.05$). As expected the RAG2-/- mice reconstituted with wild type splenocytes had recovery rates similar to C57BL/6 mice (Fig. 4:6A). Thus the attempt to recreate a C57BL/6 wild type mouse by reconstituting RAG2-/- mice with wild type splenocytes was successful.

Evidence that the reconstitution was successful in all of these experiments was seen in the examination of mice for circulating Mf. Around 50% of RAG2-/- mice used in these experiments had circulating Mf but none could be detected in the blood of the reconstituted mice (Fig. 4:6B), despite large enough sample sizes. Again around 10% of IL4-/- mice had circulating Mf (Fig. 4:6B). This data indicates that IL4 from the innate response is sufficient to prevent Mf circulating in the blood.

4:2:5 IL4 production from the innate immune response is required for protection against the establishment of L. sigmodontis *infection in C57BL/6 mice.*

We also reconstituted RAG2 / IL4 -/- mice with splenocytes from wild type C57BL/6 mice and IL4-/- mice. This experiment was terminated at day 40 post-infection and revealed no difference in the adult nematode recovery rate between any of the groups tested (Kruskal Wallis $P>0.05$) (Fig. 4:7). Therefore it appears that IL4 from the innate response is required to protect C57BL/6 mice in this experiment as mice that were reconstituted with splenocytes that were able to produce IL4 were not resistant, as would be expected if the production of IL4 from the innate was not important for

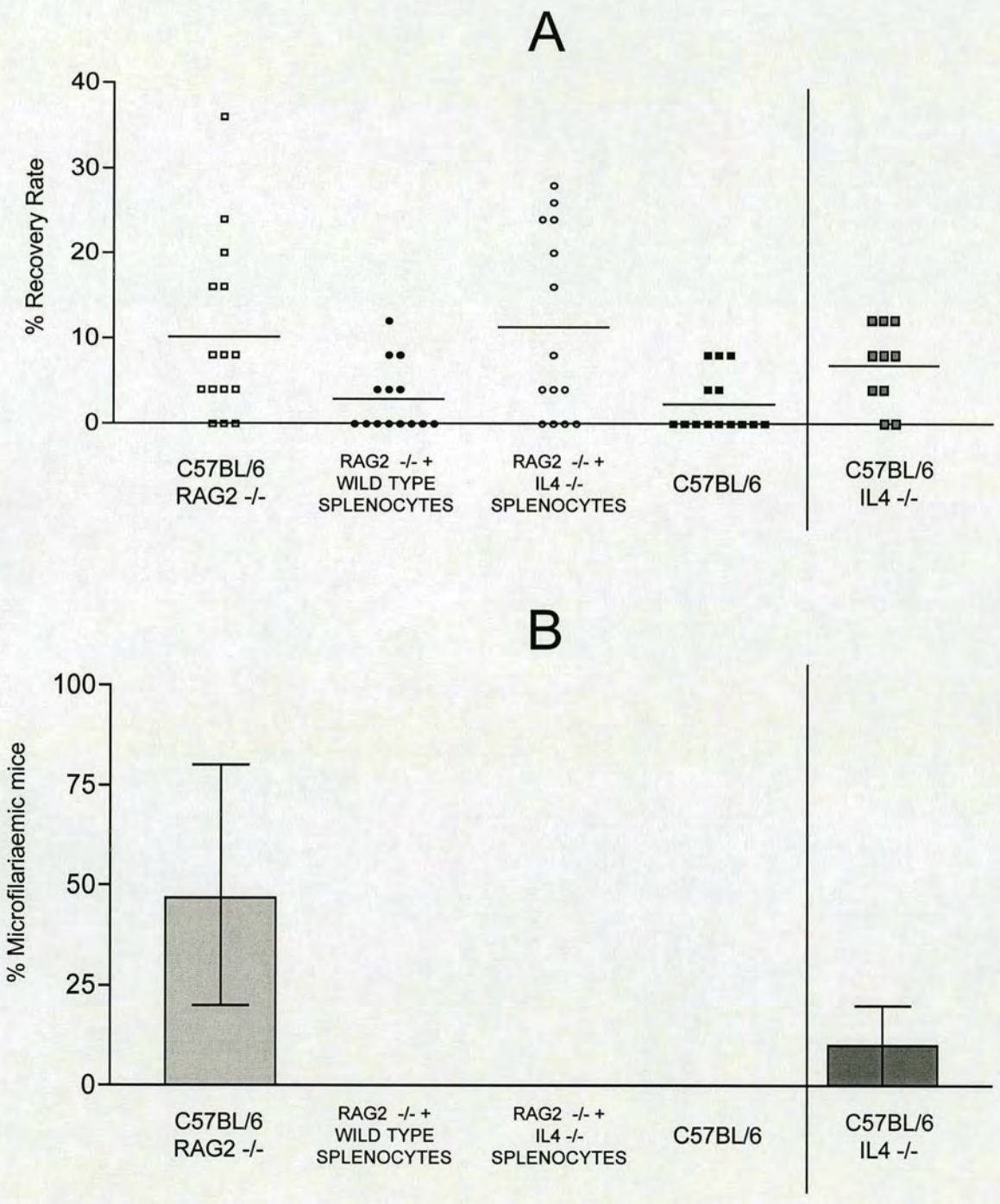


Figure 4:6. Reconstitution of C57BL/6 RAG2 -/- with wild type splenocytes restores resistance to a level similar to wild type C57BL/6 mice. Mice were reconstituted with 1 x 10^8 splenocytes and infected with *L. sigmodontis*. The % recovery rate of adult nematodes at 60 days post-infection is shown in A. Each symbol represents an individual mouse and the bars represent the mean of each group. C57BL/6 IL4-/- mice were infected as an additional control in two of the experiments. The % of mice in each group with circulating microfilariae are shown in B. The error bars represent the maximum and minimum values observed from the three different experiments carried out.

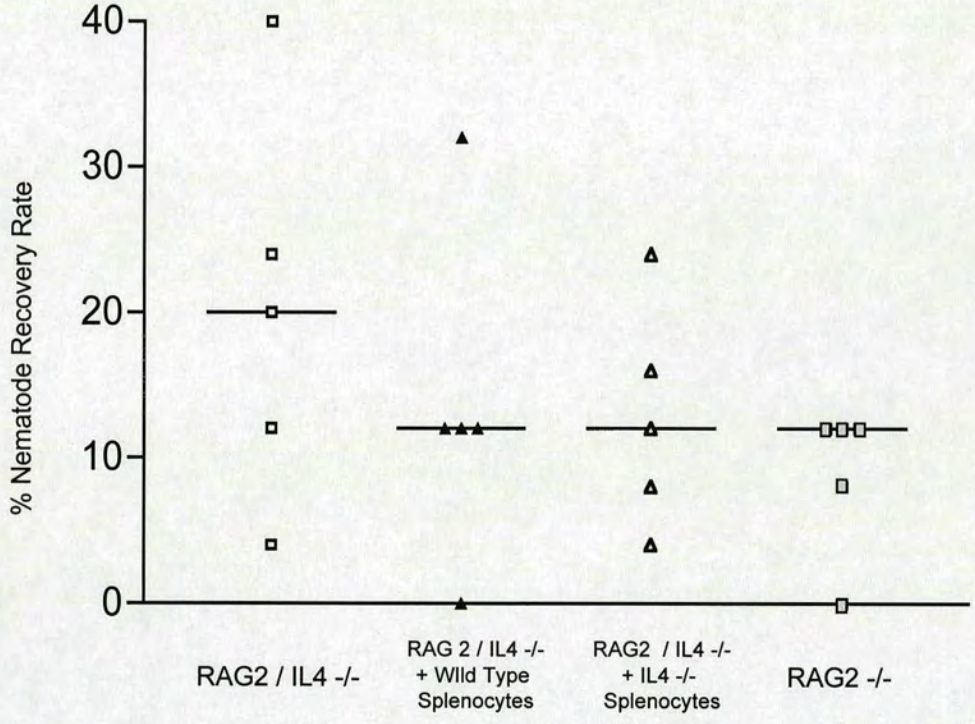


Figure 4:7. Reconstitution of RAG2 / IL4 -/- mice does not confer resistance to the establishment of infection with *L. sigmodontis.* Mice were reconstituted with 1 x 10^8 splenocytes and adult nematodes recovered at 40 days post-infection. Each infected mouse is represented by a symbol. The bars represent the median for each group.

protection. Circulating Mf do not appear in the blood until day 50 post-infection and therefore it was not possible to evaluate this parameter in this experiment.

4:4 Discussion

Filarial nematodes induce type 2 responses in both humans (Ottesen *et al.*, 1992) and in mice (Lawrence and Devaney, 2001). A hallmark of this response is the production of the type 2 cytokine IL4 and we have recently discovered this cytokine is a key factor in preventing establishment of *L. sigmodontis* infection in the resistant C57BL/6 mouse strain (Le Goff *et al.*, 2002). Immune responses arise initially from the innate arm of the immune system. This response is largely non-specific, but can influence the subsequent adaptive immune response mounted against a pathogen. The aim of this study was to determine whether innate or the adaptive immune response is more important in producing the IL4 that protects C57BL/6 mice against the establishment of infection with *L. sigmodontis*.

This study has made use of RAG2-/- mice (Shinkai *et al.*, 1992) and RAG2 / IL4 -/- mice that do not have T and B cells to mount an adaptive immune response, but can mount an innate immune response. In the case of RAG / IL4 -/- mice the innate immune response mounted will be unable to produce IL4. By reconstituting these mice with adaptive immune systems that can, or cannot produce IL4, we were able to create mice whereby only one arm of the immune system can produce IL4 upon infection with *L. sigmodontis*. By examining the establishment of the infection in

these chimeric mice it has been possible to tease apart the relative importance of IL4 from each arm of the immune response.

Surprisingly we have shown with these studies that IL4 is needed from both arms of the immune system to prevent the establishment of an infection with *L. sigmodontis* in C57BL/6 mice. RAG2-/- mice are as susceptible as IL4-/- mice (Figs. 4:3A and 4:3C) and chimeric mice that could only produce IL4 from either the innate or adaptive immune system were as susceptible as control RAG2-/- mice that were not reconstituted (Figs. 4:6A and 4:7). This indicates that IL4 production from only one arm of the immune response is equivalent to producing no IL4 at all in terms of preventing establishment of *L. sigmodontis* infection. This data highlights the ability of the innate immune response to direct the subsequent adaptive immune response and underlines a central role for the adaptive immune response in preventing the establishment of infection with *L. sigmodontis*. The importance of the IL4 from the innate response was surprising as IL4 production from T helper cells is not necessarily dependent on IL4 from other cells (Schmitz *et al.*, 1994). Other than directing adaptive immune responses against invading *L. sigmodontis* parasites, our data suggests that the innate IL4 response has a poor ability to directly protect C57BL/6 mice against the establishment of infection because RAG2-/- mice (with no adaptive immune system) were as susceptible as IL4-/- mice and RAG2 / IL4-/- mice were equally susceptible to infection as RAG2-/- mice (Figs. 4:3B and 4:6).

One criticism of the chimeric system used in these studies is that RAG-/- mice do not contain all components of the innate immune system. B1 cells are not technically part of the innate immune response as they rearrange antigen receptors using RAG. However they do participate in early immune responses (Martin and Kearney 2001) and are missing in RAG2 -/- mice. This is unlikely to be important in these studies because B cells do not appear to form an important component of the immune response against *L. sigmodontis* in mice (Martin *et al.*, 2001; Le Goff *et al.*, 2002). More importantly are certain subsets of T cells such as NK T cells or γδ-T cells that are also considered to form part of the innate immune response, yet are missing in RAG2 -/- mice. The contribution of γδ-T cells in filarial infection is currently unreported, however NK T cells have been shown to be an important source of innate system IL4 shortly after infection with *B. pahangi* (Balmer and Devaney 2001). Experiments reconstituting C57BL/6 IL4-/- mice with NK T cells from wild type mice would address the importance the IL4 evoked from this cell type in protecting C57BL/6 mice from establishment with *L. sigmodontis* infection.

No circulating Mf were detected in any of the chimeric mice that could produce IL4 from the innate system, regardless of the IL4 status of the reconstituted adaptive immune system (Fig. 4:6B). A previous study with *L. sigmodontis* indicated that IL4 damages the fecundity of adult *L. sigmodontis* (Volkmann *et al.*, 2003). Indeed in this study circulating Mf were observed in the blood of IL4-/- mice but not C57BL/6 wild type mice (Figs. 4:3B and 4:6B). We observed few effects of IL4 from the innate system of chimeric mice on the establishment of adult filarial nematodes (Fig.

4:6A), and yet observed no circulating Mf in these mice. Therefore it is possible that IL4 from the innate immune system does have effects on the fecundity of adult nematodes.

It is also possible that IL4 enhances the ability of cells of the innate system that may participate in Mf clearance. This seems unlikely since it has been shown that Mf injected directly into mice deficient in IL4 are cleared at an equivalent rate to that in wild type mice (Volkmann *et al.*, 2003). An alternative explanation for this data is that IL13, a cytokine that can compensate for IL4, is still operational in these chimeric mice. However infections of mice deficient in the IL4 receptor α-chain (IL4R -/-), a protein also shared by the IL13 receptor (Brombacher 2000), indicate that IL13 does not play significant role in adult establishment, nor Mf clearance from the blood (Volkmann *et al.*, 2003). Contrary to these results we have observed a decreased clearance of Mf from the bloodstream of IL4R-/- mice on the same background as the Volkmann study (2003) (Le Goff *et al.*, unpublished observations). *B. malayi* infections of mice deficient in IL13 have also indicated that IL13 may participate in the clearance of Mf (Lawrence and Devaney 2001).

Further studies are now needed, using the chimeric system to identify cells types that may be involved in producing the IL4 that prevents the establishment of *L. sigmodontis* infection. To identify cells of the immune response that produce IL4 in the innate system, mixed bone-marrow chimeric mice could be generated whereby only specific cells of the innate system such as NK T cells (Balmer and Devaney,

2001) or macrophages and dendritic cells can produce IL4. Subsequent secondary reconstitution of these mice with wild type splenocytes would determine which cell types produce IL4 that is able to instruct the adaptive immune system to protect mice against the establishment of *L. sigmodontis* infection. An alternative approach to the problem is to generate mice that are IL4 – deficient only in specific cell types using the cre-lox system (for example see De Boer *et al.*, 2003), and look for susceptibility to the establishment of *L. sigmodontis* infection.

Likewise RAG2-/- mice could be reconstituted with splenocytes whereby only the T cells or B cells can produce IL4 to determine the important source of IL4 from the adaptive immune system. It is hypothesised that T cells are the important source of IL4 from the adaptive immune system because ablation of CD4+ cells has been linked with higher *L. sigmodontis* burdens, and a concomitant decrease in the anti-filarial type 2 response in BALB/c mice (Al-Qaoud *et al.*, 1997). On the other hand B cells appear to play very little role in the resistance (Le Goff *et al.*, 2002). Despite the finding that B cells can be an important source of IL4 in for the establishment of a type 2 response in general (Stockinger *et al.*, 1996; Johansson-Lindbom and Borrebaeck, 2002) this does not appear to be an essential mechanism in the production of the IL4 to protect C57BL/6 mice against establishment of *L. sigmodontis* infection.

In conclusion these studies have indicated that IL4 production from both the innate and acquired arms of the immune response is necessary to protect C57BL/6 mice

against the establishment of infection with *L. sigmodontis*. Additionally we have demonstrated the usefulness of chimeric models in dissecting protective immune responses evoked against parasites.

CHAPTER 5

Malaria / filaria co-infection can lead to an acceleration of adult filarial nematode death

5:1 Introduction

The geographical distribution of filarial infection overlaps with that of many other infections (Fig. 5:1). Therefore it can be inferred that many people infected with filarial nematodes will also be infected with other pathogens. Observations of the impact of co-infection on filarial nematode infection are largely anecdotal, and researchers have generally attempted to control for the co-infected individuals in field studies rather than study them. However the immune responses mounted against co-infecting pathogens may interact with the immune responses that an individual mounts to filarial nematodes. Since anti-filarial immune responses are a key factor contributing towards the outcome of filarial infection (Maizels *et al.*, 1991), immune responses generated by co-infecting pathogens may potentially be a very important factor in the interpretation of field studies into, and an important determinant of the outcome of, filarial nematode infection.

Malaria is an infection that is widespread in countries of the developing world (World Health Organisation, 2002). Co-infection with malaria and filarial nematodes do occur (Piessens *et al.*, 1983; Guderian *et al.*, 1991; Ghosh and Yadav, 1995; Ravindran *et al.*, 1998; Chadee *et al.*, 2003) but possible within-host interactions between these two

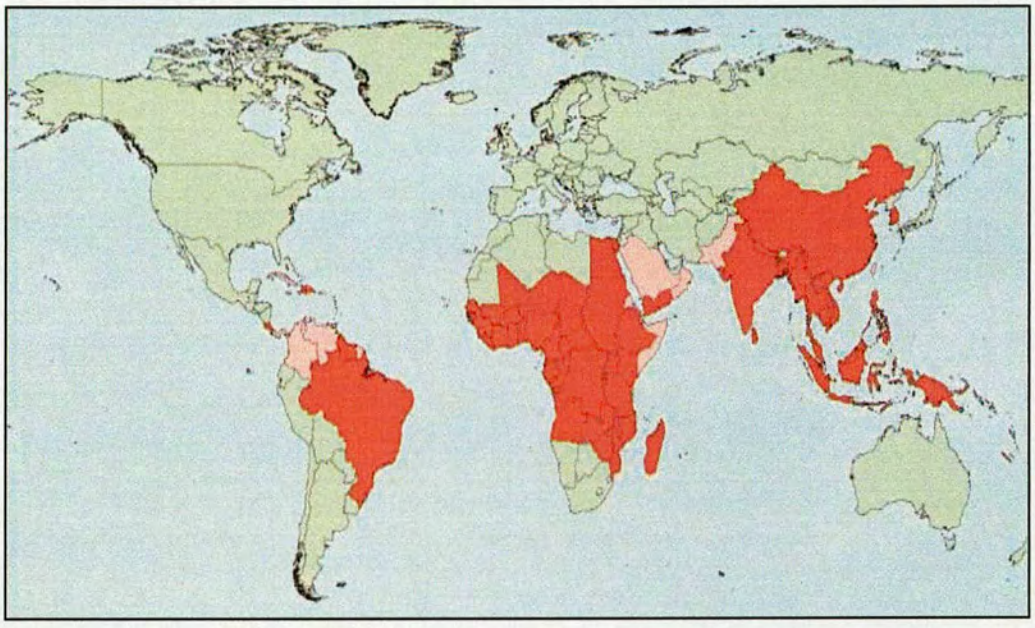


Figure 5:1. The geographical distribution of lymphatic filariasis (World Health Organisation, 2000; http://www.who.int/ctd/filariasis/library/countries3.html). The areas shown in red represents areas of high endemicity, the areas shown in pink represent areas where the endemicity in unknown and the areas shown in green represent non-endemic areas.

parasites have not been studied. Evidence from analyses of other helminth and malaria co-infections support the hypothesis that co-infection is an important factor to consider in parasitic diseases. Individuals in Thailand co-infected with intestinal helminths and malaria were shown to interact in such a way that altered malaria-induced pathology (Nacher *et al.*, 2002a; Nacher *et al.*, 2002b) and malaria parasite load (Nacher *et al.*, 2001b; Nacher *et al.*, 2002c). Co-infection studies in murine models have found effects upon the dynamics of both malaria infection (Yan *et al.*, 1997; Helmby *et al.*, 1998; Yoshida *et al.*, 2000) and helminth infection (Phillips *et al.*, 1974; Phillips and Wakelin, 1976; Modric and Mayberry, 1994).

In this study we have used parasitic infections of *Litomosoides sigmodontis* and *Plasmodium chabaudi chabaudi* in laboratory mice to examine the infection dynamics and immunological interactions that may occur during co-infection with filarial nematodes and malaria. More specifically our aim was to determine whether co-infection with *P. chabaudi* altered the ability of established *L. sigmodontis* adult nematodes to survive in BALB/c mice. *L. sigmodontis* migrates to the thoracic cavity, induces a strong type 2 response, characterised by the cytokines IL4, IL5 and IL10 (Marechal *et al.*, 1997) and begins to produce microfilariae (Mf) approximately 50 days post-infection (for review see Hoffman *et al.*, 2000). *P. chabaudi* multiplies asexually within the mouse bloodstream (Fig. 5:2) and peaks in density between days 5 and 7 post-infection leading to a low level (and almost undetectable) chronic infection from day 14 -18 post-infection. (Langhorne *et al.*, 2002). Initially *P. chabaudi* evokes a strong inflammatory response typified by the type 1 cytokine

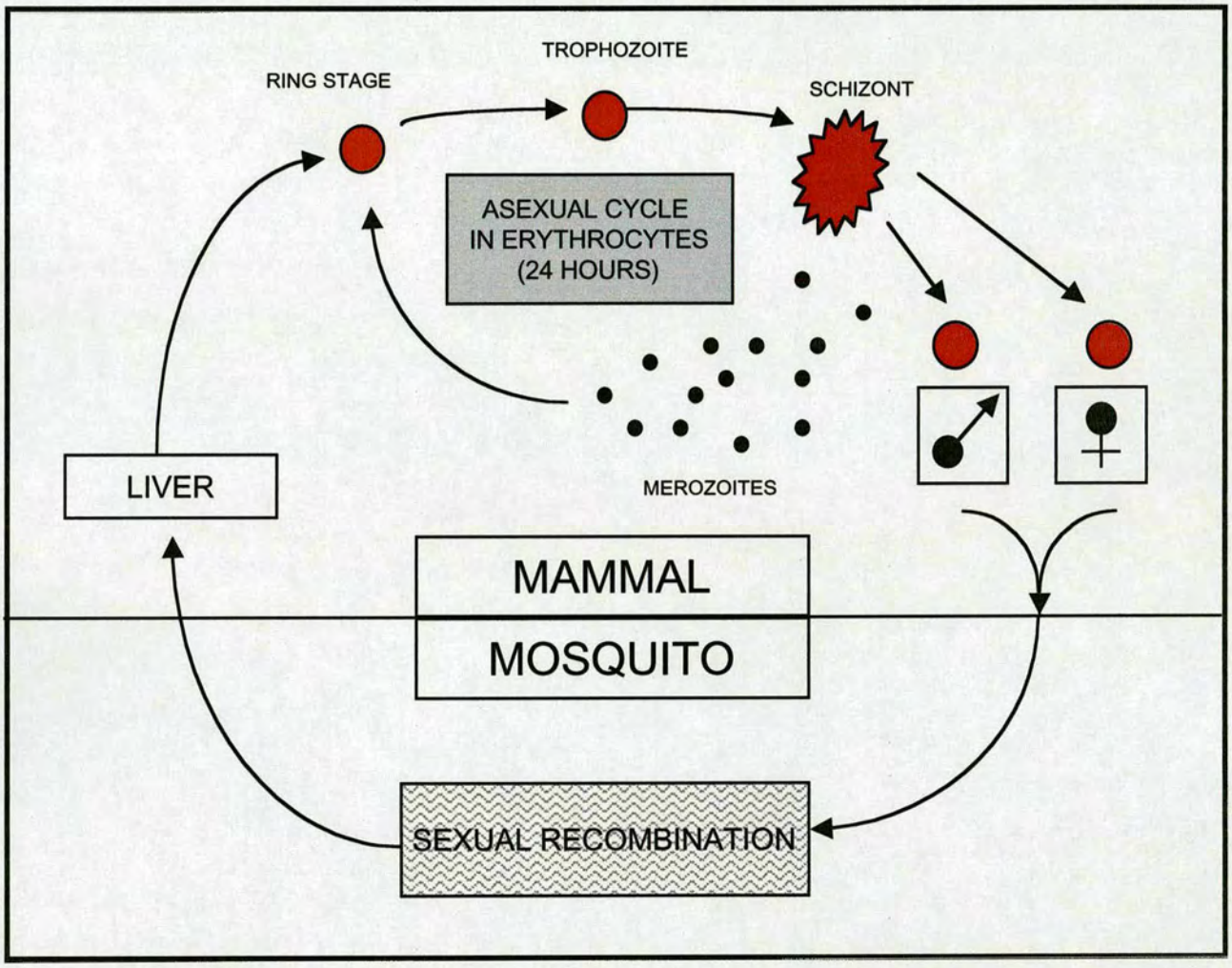


Figure 5:2. The life cycle of *Plasmodium chabaudi*.

interferon-γ (IFNγ) (Langhorne *et al.*, 1989; Langhorne and Simon, 1989) and later in infection, begins to additionally induce type 2 associated cytokines (von der Weid *et al.*, 1994; Stevenson and Tam, 1993).

We have found that the presence of malaria infection may accelerate the death of adult *L. sigmodontis* parasites. This finding was associated with anti-filarial immune responses that were less type 2 skewed than in singly infected animals. The effects of *L. sigmodontis* on the infection dynamics of *P. chabaudi* were less clear, although there was evidence that anti-malaria immune responses were also altered in co-infected animals when compared to singly infected animals.

5:2 Results

5:2:1 P. chabaudi *co-infection can accelerate the death of adult* L. sigmodontis.
We infected BALB/c susceptible mice with *L. sigmodontis*, and at day 60 post-infection, when approximately half of the mice have detectable circulating Mf (Petit *et al.*, 1992), we challenged with a *P. chabaudi* infection (Fig. 5:3). After 20 days of malaria infection we examined the number of surviving adult parasites in singly and co-infected animals (Fig. 5:4A and 5:4B). These experiments utilized large sample sizes and were performed twice. The number of adult nematodes recovered was not different between the co-infected and singly infected group in experiment 1 (One-tailed Mann-Whitney U-Test, $P>0.05$) (Fig. 5:4A) but was lower in the co-infected group in the second experiment (One-tailed Mann-Whitney U-Test, $P<0.05$) (Fig.

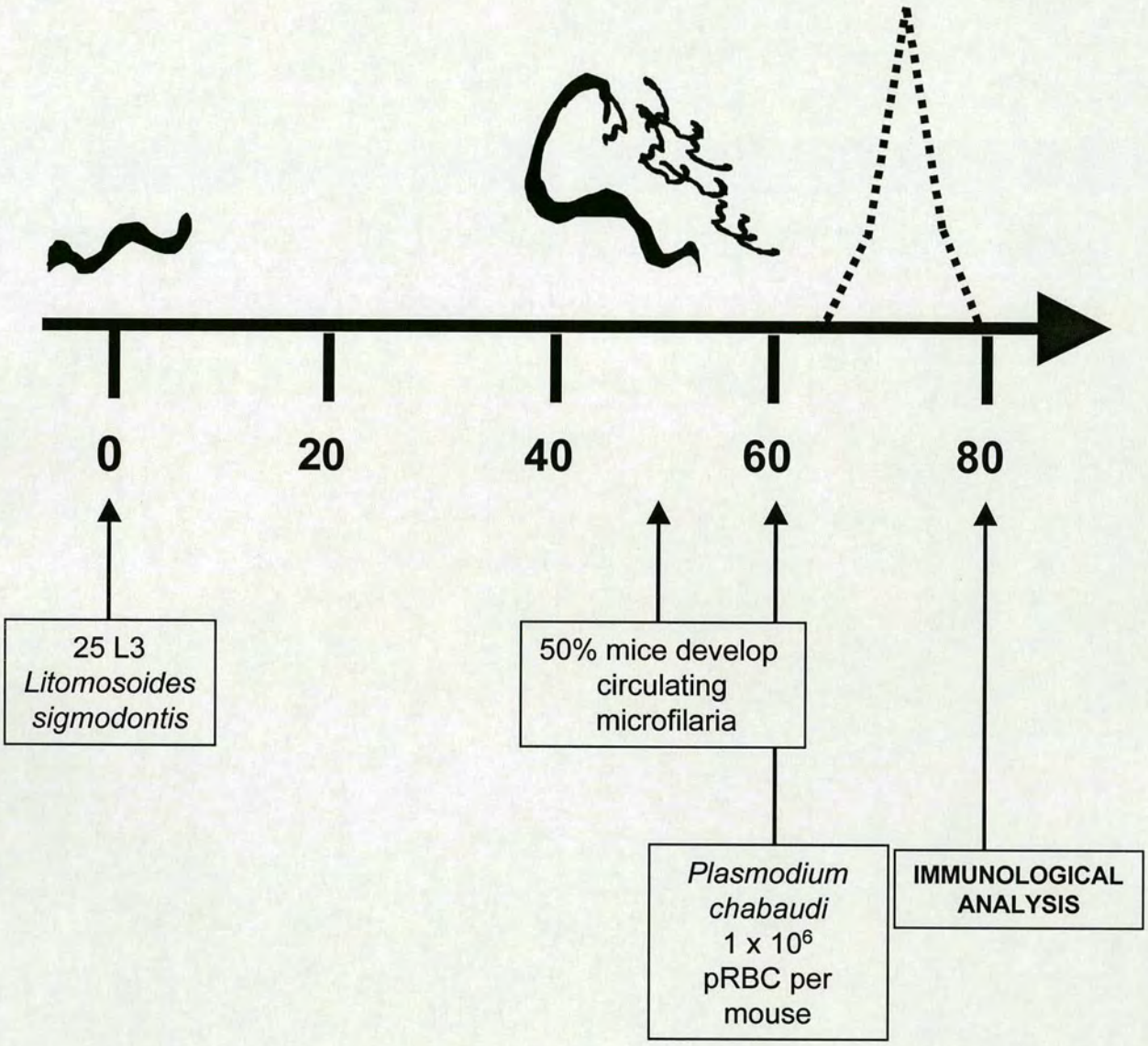
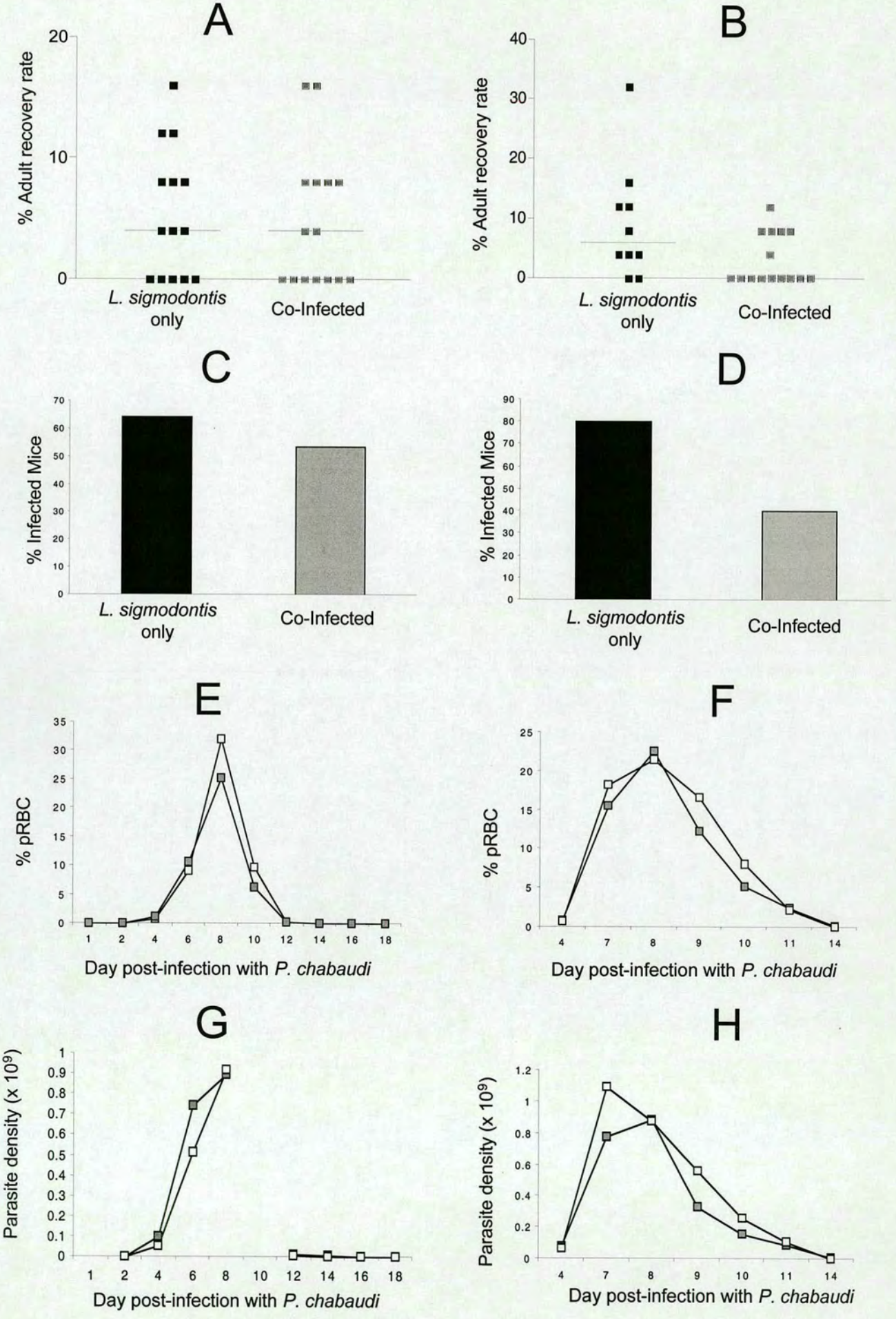


Figure 5:3. *L. sigmodontis* / *P. chabaudi* co-infection protocol. BALB/c mice were infected with 25 *L. sigmodontis* L3 on day 0. At day 60 when some of the infections have become patent, the mice were challenged with 1 x 10^6 *P. chabaudi* AS. Malaria infection was monitored at regular intervals and the immune responses of all mice were examined at day 80 post-infection with *L. sigmodontis* (day 20 post-infection with *P. chabaudi).*

Figure 5:4 The parasitology of co-infection experiments with *L. sigmodontis* and *P. chabaudi*. 2 separate experiments are shown for comparison. The first experiment is shown in A,C,E and G. The second experiment is shown in B,D,F and H.
A and B represent the number of *L. sigmodontis* adult parasites recovered at day 80 post-infection in the thoracic cavity as a % of the initial L3 innoculum used to set up the infection. The black squares represent individual animals infected with *L. sigmodontis* only. The grey squares represent animals co-infected with *L. sigmodontis* and *P. chabaudi*. The bars represent the median % recovery rate in each group. C and D represent the % of mice that harboured adult parasites at day 80 post-infection. The black bars represent group infected with *L. sigmodontis* only. The grey bars represent the animals co-infected with *P. chabaudi*. E and F represent the % of red blood cells containing *P. chabaudi* parasites over the time of *P. chabaudi* infection (days 60-80 post infection with *L. sigmodontis).* G and H represent the malaria parasite density over the time of *P. chabaudi* infection. A broken coulter counter led to the missing points on day 10 of graph G. In E,F G and H the white squares represent the group mean of mice infected with *P. chabaudi* only. The grey squares represent the group mean of the mice co-infected with *L. sigmodontis*. Error bars have been omitted for clarity.


A
% Adult recovery rate
20
10
0
L. sigmodontis only
Co-Infected
B
% Adult recovery rate
40
30
20
10
0
L. sigmodontis only
Co-Infected
C
% Infected Mice
70
60
50
40
30
20
10
0
L. sigmodontis only
Co-Infected
D
% Infected Mice
90
80
70
60
50
40
30
20
10
0
L. sigmodontis only
Co-Infected
E
% pRBC
35
30
25
20
15
10
5
0
1 2 4 6 8 10 12 14 16 18
Day post-infection with P. chabaudi
F
% pRBC
25
20
15
10
5
0
4 7 8 9 10 11 14
Day post-infection with P. chabaudi
G
Parasite density (x 10⁹)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1 2 4 6 8 10 12 14 16 18
Day post-infection with P. chabaudi
H
Parasite density (x 10⁹)
1.2
1
0.8
0.6
0.4
0.2
0
4 7 8 9 10 11 14
Day post-infection with P. chabaudi

5:4B). The percentage of mice harbouring live *L. sigmodontis* adult parasites at day 80 post-infection was lower in the co-infected group than in the singly infected group in both experiments (Fig. 5:4C and 5:4D) but this trend was only significant in the second experiment (Logistic Regression, P=0.05). Thus there was little effect of a malaria co-infection in the first experiment, but the second experiment suggests that malaria co-infection accelerated the death of adult nematodes.

5:2:2 L. sigmodontis *can decrease* P. chabaudi *parasitaemia.*

The course of malaria infection was closely monitored over the course of infection in both experiments. In both cases the % of red blood cells that were parasitised by *P. chabaudi* (pRBC) in co-infected animals was equal to or lower than in the group singly infected with *P. chabaudi* (Figs. 5:4E and 5:4F). The peak % pRBC at day 8 post-infection was significantly lower in co-infected animals compared with singly infected animals in the first experiment (Mann Whitney U-test, $P<0.05$) when no significant differences were observed in nematode recovery rate (Figs. 5:4E and 5:4A). Interestingly, there was no difference in the peak % pRBC in the second experiment (Mann Whitney U-test, $P>0.05$) when co-infection appeared to accelerate the death of adult nematodes (Figs. 5:4F and 5:4B).

Parasite density was calculated from the red blood cell density and pRBC measurements at each time point. The peak parasite density in co-infected animals was the same as singly infected animals in the first experiment (Day 8 post-malaria infection, Mann Whitney-U test, $P>0.05$) despite the statistically lower proportion of

pRBC in this group of animals at this time point (Figs. 5:4G and 5:4E). In the second experiment the parasite density in singly infected animals peaked 1 day earlier (day 7 post-malaria infection) than in the first experiment (day 8 post-malaria infection) (Fig. 5:4H). Nevertheless, in this experiment peak parasite density in co-infected animals (measured at day 8 post-infection in the co-infected group) was statistically less than the peak parasite density in singly infected animals (Mann Whitney-U test, $P<0.05$). Overall in the first experiment co-infection with *L. sigmodontis* was associated only with a decreased peak in the % of pRBC. On the other hand in the second experiment, acceleration in filarial nematode death was coupled with a decrease in peak malaria parasite number.

*5:2:*3 L. sigmodontis *had little effect on the pathology induced by* P. chabaudi *infection*

During the course of malaria infection, we monitored the amount of malaria induced weight loss and anaemia in singly and co-infected mice. The trend in weight loss was different in the two experiments. In the first experiment the co-infected animals appeared to lose less weight than the singly infected animals overall (Fig. 5:5A). This trend was reversed in the second experiment (Fig. 5:5B). The maximum weight loss for each group of animals occurred at day 10 post-malaria infections but was not statistically different in either experiment (Experiment 1, Mann Whitney-U test, $P=0.075$; Experiment 2, Mann Whitney-U test, $P=0.278$). The maximum malaria-induced anaemia occurred at day 8 post-malaria infection in experiment 1 and day 9 post-malaria infection in experiment 2, and again was not different between singly

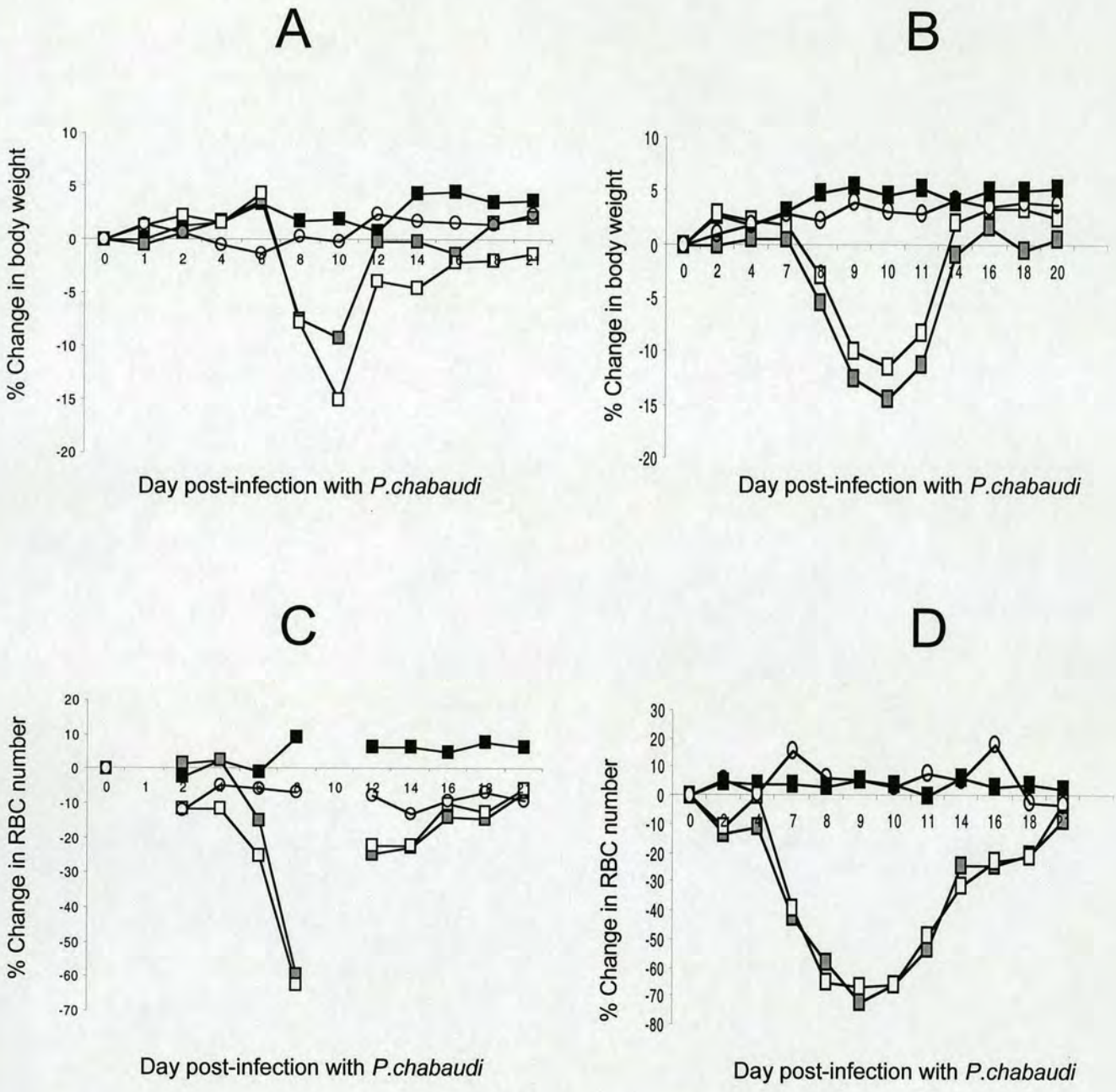


Figure 5:5. The pathology induced by *P. chabaudi* in animals co-infected with *L. sigmodontis*. The data shown represent two separate experiments. The first experiment is shown in A and C. The second experiment is shown in B and D. Figures A and B show the % of body weight lost gained by mice over the time of infection with *P chabaudi*. Figures C and D show the % of red blood cells lost and gained by mice over the time of infection with *P. chabaudi*. A broken coulter counter led to the missing points on day 10 of graph C. In all figures the white squares represent the group mean of animals infected with *P. chabaudi* only. The grey squares represent the group mean of animals co-infected with *L. sigmodontis*. For comparison the group means of animals infected with *L. sigmodontis* and uninfected animals are shown by the black squares and white circles respectively. Error bars showing the maximum and minimum values for each group at each time point have been omitted for clarity.

and co-infected groups in either experiment (Experiment 1, Mann Whitney-U test, P=0.740; Experiment 2, Mann Whitney-U test, P=0.168). Therefore there were no detectable differences in host pathology induced by malaria infection between singly and co-infected animals.

5:2:4 Non-specific immune responses were less type 2 skewed in co-infected mice compared to mice singly infected with L. sigmodontis.

The immune responses of singly and co-infected mice were measured at day 80 post-*L. sigmodontis* infection. The splenocytes of mice were non-specifically stimulated with Con A and the number of cells secreting the type 1 cytokine IFNγ, and the type 2 cytokine interleukin-4 (IL4), was measured using ELISPOT. In all cases the number of cells secreting IFNγ and IL4 were positively correlated for all groups of mice (ANOVA $P<0.01$) (Fig. 5:6). There were no differences in the number of cells secreting IFNγ between the different groups of animals in either experiment (ANOVA both $P>0.05$). This was also true for the number of cells secreting IL4 (ANOVA both $P>0.05$). In both experiments the splenocytes of co-infected mice appeared to be less skewed towards the axis representing IL4 secreting cells compared to the mice singly infected with *L. sigmodontis* (Fig. 5:6). Surprisingly the mice singly infected with *P. chabaudi* were not skewed towards the axis representing IFNγ secreting cells (Fig. 5:6) as might be expected from a type 1 inducing pathogen. Although the skew of the non-specific immune response induced by Con A was similar in both experiments, the number of IL4 –secreting cells, or magnitude of type 2 responsiveness, was approximately four fold in the second experiment when there

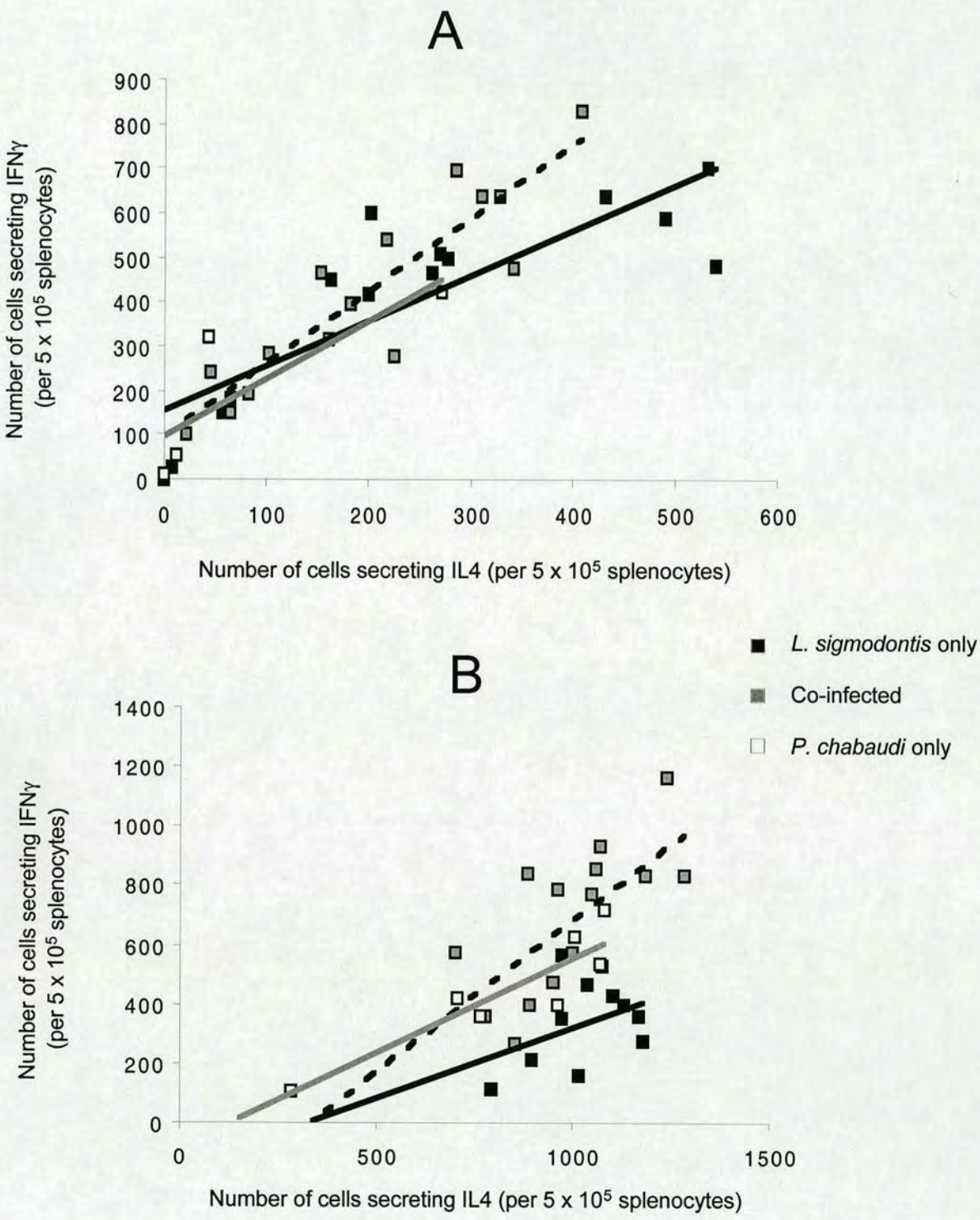


Figure 5:6. Non-specific Con A induced cytokine responses from splenocytes of singly-infected and co-infected mice at day 80 post-infection. The graphs show the number of cells that secreted IL4 plotted against the number of cells that secreted IFNγ. A and B represent the results from two separate experiments.The black squares represent the responses individual animals singly infected with *L. sigmodontis*.The grey squares represent co-infected animals. The white squares represent animals infected with *P. chabaudi* only. The lines shown are the least square best fit lines for each group. The singly infected animals are represented by the black line (*L. sigmodontis*) and grey line (*P. chabaudi*). The co-infected animals are represented by the dashed line.

was an acceleration in the death of adult filarial nematodes, and a concomitant decrease in malaria parasite density in co-infected animals (Fig. 5:6B).

5:2:5 Co-infected animals mount parasite-specific antibody responses that are less polarised than those of singly infected animals.

As an additional measure of immune bias we examined levels of pathogen-specific antibodies. We measured IgG2a as an indication of the level of anti-parasite type 1-associated immune responses and IgG1 as a measure of type 2-associated immune responses. Antibody responses were analysed by Kruskal Wallis including both groups of singly infected animals and the co-infected animals, followed by Dunn's pairwise multiple comparison test. As expected from infections with type 2 inducing parasites, animals singly infected with *L. sigmodontis* induced anti-filarial antibody responses that were dominated by the isotype IgG1 (Figs. 5:7A and 5:7C). The level of IgG1 secreted by co-infected animals was not different from animals singly infected with *L. sigmodontis* (Dunn's Pairwise Multiple Comparison test $P>0.05$ in both experiments). (Figs. 5:7A and 5:7C). However in the first experiment, but not the second experiment, co-infected animals had increased levels of circulating anti-*L. sigmodontis* IgG2a compared to animals singly infected with *L. sigmodontis* (Experiment 1, Dunn's multiple pairwise comparison test $P<0.001$; Experiment 2, Kruskal Wallis $P>0.05$) (Fig. 5:7A). However this increased level of IgG2a was not above the level of anti-filarial IgG2a cross-reactive antibodies observed in animals singly infected with *P. chabaudi* (Dunn's multiple pairwise comparison test $P>0.05$).

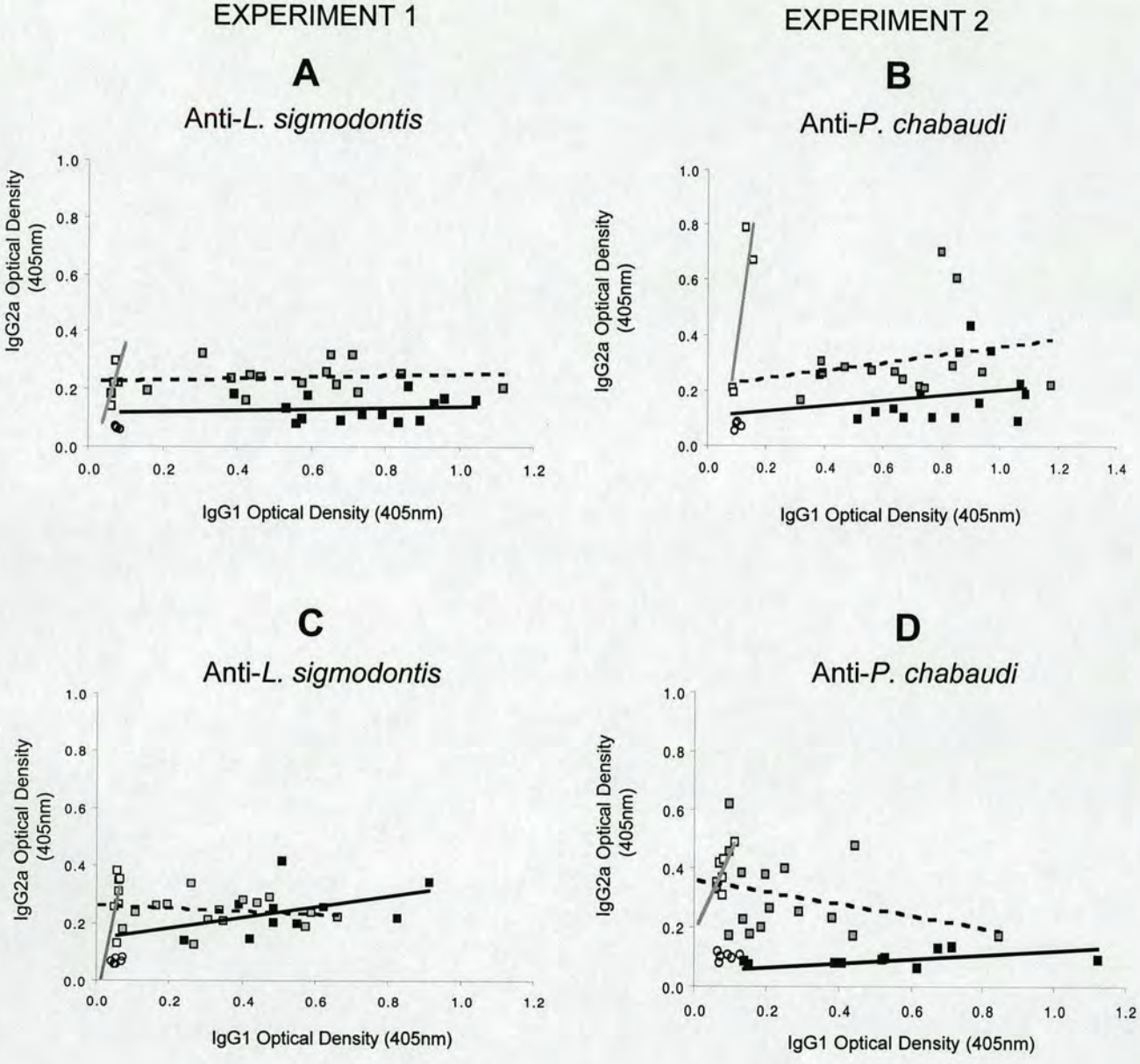


Figure 5:7 Antibody responses to *L. sigmodontis* and *P. chabaudi* in singly-infected and co-infected animals at day 80 post-infection. Type 1 induced IgG2a is represented on the y-axis and type 2 induced IgG1 is represented on the x-axis. Sera was diluted 1:800 to detect anti-*L. sigmodontis* antibodies and 1:100 to detect anti-*P. chabaudi* antibodies. Two experiments are depicted. The first experiment is represented by A and B. The second experiment is represented by C and D. A and C show responses against *L .sigmodontis*. B and D show responses against *P. chabaudi.* In all cases the black squares represent individual animals infected with *L. sigmodontis* only, the white squares represent individual animals infected with *P. chabaudi* only and the grey squares represent individual animals that are co-infected.Responses of naive animals are shown by white circles. The lines represent the least square best fit lines through the data points for mice in each group. The singly infected animals are represented by the black line (*L. sigmodontis*) and grey line (*P. chabaudi*). The co-infected animals are represented by the dashed line.

Therefore this increase could be explained by antibody cross-reactivity, rather than a change in skew of the anti-parasite immune response.

The antibody response against *P. chabaudi* in singly –infected animals was dominated by the type 1 associated isotype IgG2a (Figs. 5:7B and 5:7D). In co-infected animals the level of IgG2a was similar to animals singly infected with *P. chabaudi* (Dunn's pairwise multiple comparison test $P>0.05$ in both experiments). However co-infected animals additionally mounted an anti-*P. chabaudi* IgG1 response that was absent *P. chabaudi* singly infected animals (Dunn's pairwise multiple comparison test $P<0.05$ in both cases). This anti-*P. chabaudi* IgG1 response was not greater that the level of cross-reactive IgG1 antibody measured in animals singly infected with *L. sigmodontis* (Dunn's pairwise multiple comparison test $P>0.05$ in both cases). Therefore, similarly to the anti-*L. sigmodontis* response in co-infected animals, the additional anti-*P. chabaudi* IgG1 response observed in co-infected animals may be due to cross-reactivity, rather than a skew in the anti-parasite immune response.

The co-infection protocol we have used for these experiments dictates that *P. chabaudi* is infecting animals that have already been infected with *L. sigmodontis* for 60 days. Therefore the malaria parasites have to evoke responses in an environment that is already strongly biased towards type 2. To assess the impact of a type 1 response inducing *P. chabaudi* on overall the overall type 2 response established by *L. sigmodontis* infection we measured the total circulating polyclonal IgE levels from

animals in the first experiment. The production of this antibody isotype is highly dependent on the type 2 signature cytokine IL4. Additionally *P. chabaudi* primary infections do not induce IgE (Helmby *et al.,* 1996) as we have observed here (Mann-Whitney U-test P>0.05) (Fig. 5:8). This isotype can therefore be used as an additional measure of the anti-filarial type 2 response. IgE OD values were multiplied by 100 and logarithmically transformed prior to statistical analysis. There appeared to be down-regulation of IgE in co-infected animals compared to animals singly infected with *L. sigmodontis*, although this difference was not statistically significant (Student's t-test, P=0.13) (Fig. 5:8).

5:2:6 Co-infection did not alter immune responses in mice with circulating microfilariae.

In BALB/c mice 50% of infections with *L. sigmodontis* become patent with circulating microfilariae (Mf+) (Petit *et al.*, 1992). Because Mf and malaria share the same compartment in the mouse (blood), we tested whether mice that were Mf+ mounted different responses in singly and co-infected animals. In both of the experiments, we did not find any statistical differences in the anti-*L. sigmodontis* IgG1 antibody responses mounted amongst co-infected and singly infected *L. sigmodontis* animals that were Mf+ and Mf- (Kruskal Wallis, P>0.05 in both cases) (Figs. 5:9A and 5:9B). There was a trend towards a higher production of anti-filarial IgG2a in MF+ mice in the mice singly infected with *L. sigmodontis* in both experiments although this was not statistically significant (Dunn's pairwise multiple comparison test P>0.05 in both cases) (Figs. 5:9C and 5:9D). This trend was also

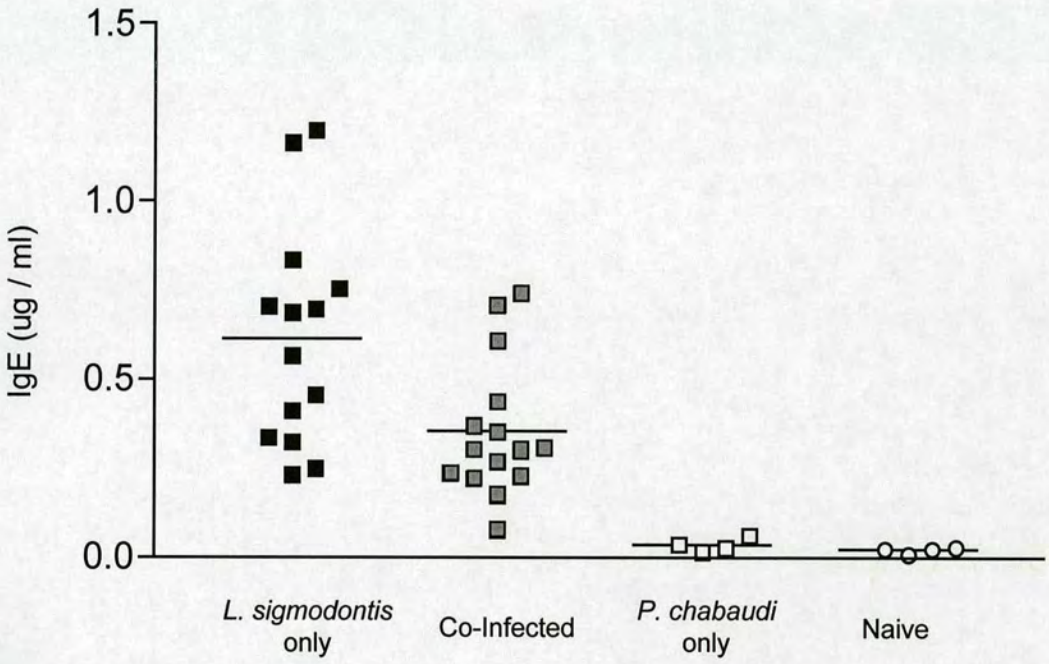


Figure 5:8 Total circulating IgE in the serum at day 80 post-infection. Each square represents an individual animal. Sera was diluted 1:20. The black squares represent animals singly infected with *L. sigmodmontis*, the white squares represent animals singly infected with *P. chabaudi* and the grey squares represent animals that are co-infected. Naive animals (shown by the white squares) are shown for comparison. The bars show the mean of each group.

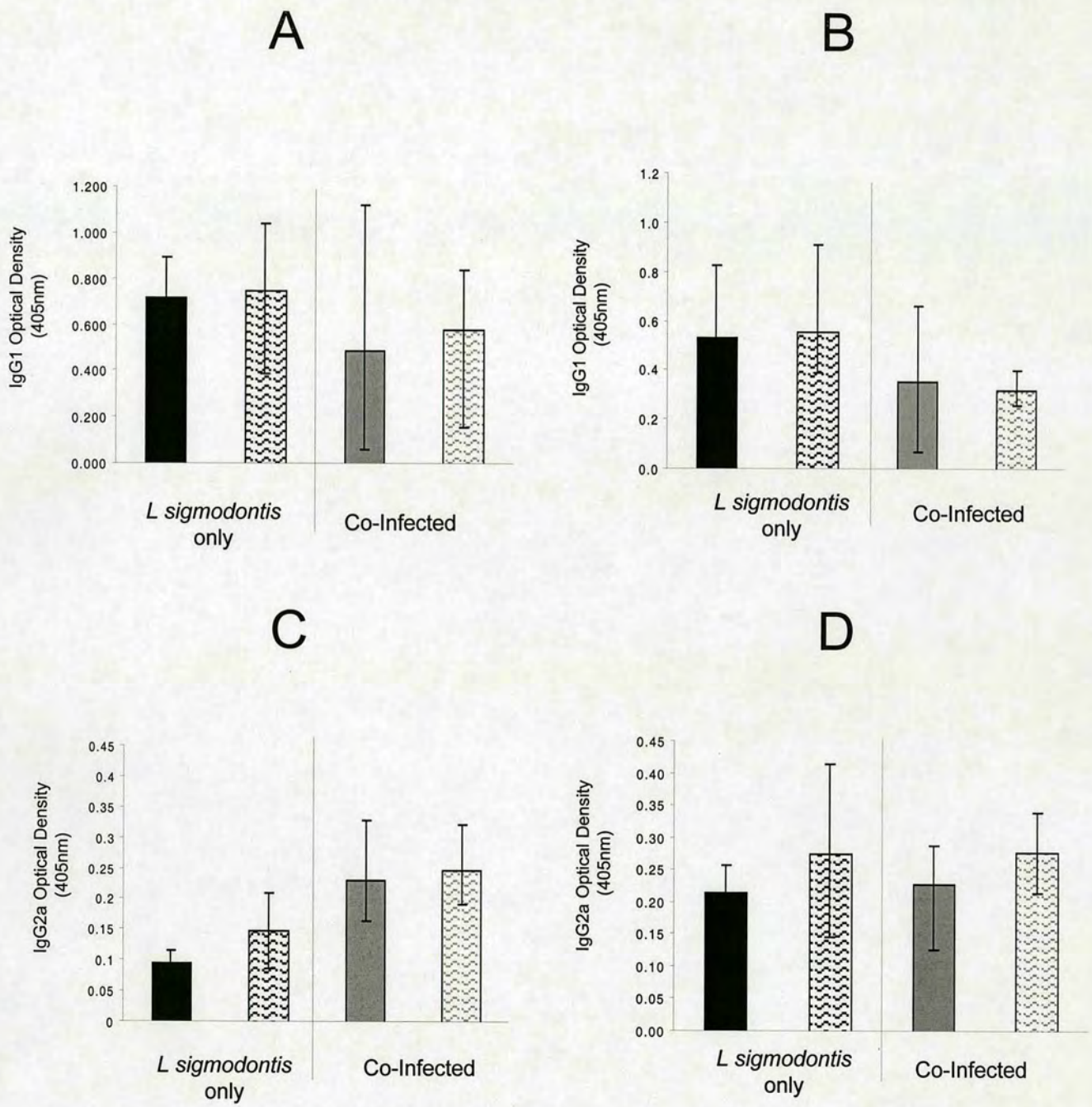


Figure 5:9. Antibody responses against *L. sigmodontis* in animals singly infected with *L. sigmodontis* with (black dashed bars) and without circulating Mf (black bars) and co-infected animals with (grey dashed bars) and without (grey bars) circulating Mf.Sera was diluted 1:800. Error bars represent the maximum and minimum values. Two experiments are shown. The first experiment is shown by A and C and the second experiment is shown by B and D. IgG1 is shown in A and B and IgG2a is shown in C and D.

observed in Mf+ co-infected animals in the second experiment, but again was not significant (Kruskal Wallis P>0.05). This is consistent with the known ability of Mf to evoke a type 1 response (Lawrence *et al.*, 1994).

5:2:7 P. chabaudi *and* L. sigmodontis *contain molecules with similar epitopes that result in the production of cross-reactive antibodies.*

A surprising observation in our studies was the finding of anti-*P. chabaudi* antibodies in animals singly-infected with *L. sigmodmontis* that were of the IgG1 isotype (Figs. 5:7B and D). Similarly we observed anti-filarial IgG2a antibodies in two animals singly infected with *P. chabaudi* (Fig. 5:7B). We ran western blots with sera from animals selected from the first experiment to further examine the cross-reactive nature of the antibodies produced in each infection. By this technique we were able to demonstrate that IgG1 antibodies produced in filarial infection reacted with molecules of the same size in *P. chabaudi* infection (Fig. 5:10B, lanes 1 and 2). Additionally these antibodies produced in the sera of this animal reacted with two bands that appeared to be specific to *P. chabaudi* that were approximately 70 kDa and 45 kDa in size (Fig. 5:10B lane 2). We also observed the production of antibodies against red blood cell proteins (Fig. 5:10B lane 3). Interestingly the co-infected animal we examined produced more IgG1 antibodies to *P. chabaudi* than to *L. sigmodontis* (Fig. 5:10D, lanes 1 and 2) even though all anti-*P. chabaudi* bands may be an artefact of cross-reactivity induced by *L. sigmodontis* infection (Fig. 5:10B, lane 2). IgG1 antibodies were absent in the animal singly infected with *P. chabaudi* (Fig. 5:10C). Surprisingly, IgG1 antibodies against *L. sigmodontis*

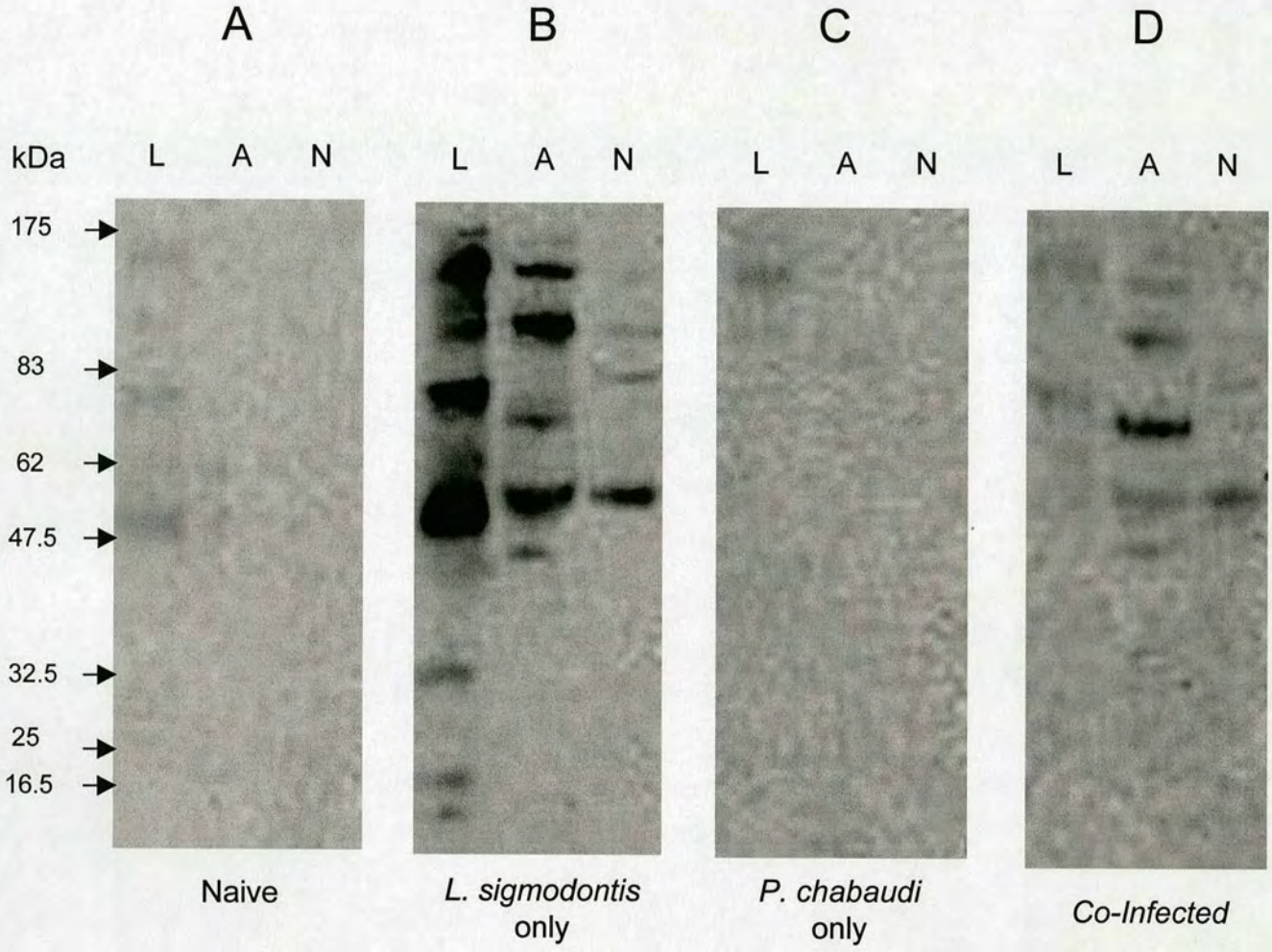


Figure 5:10. Western blot analysis of IgG1 antibody responses in mice infected with *L. sigmodontis*, *P. chabaudi* and animals co-infected with the two parasites. Each blot represents one animal from each group. A blot with sera from a naive animal is shown for comparison. Lanes marked L are loaded with *L. sigmodontis* adult antigen, lanes marked A are loaded with *P.chabaudi* infected red blood cell antigen and lanes marked N are lanes loaded with naive blood cell antigen as a control for red blood cell proteins.

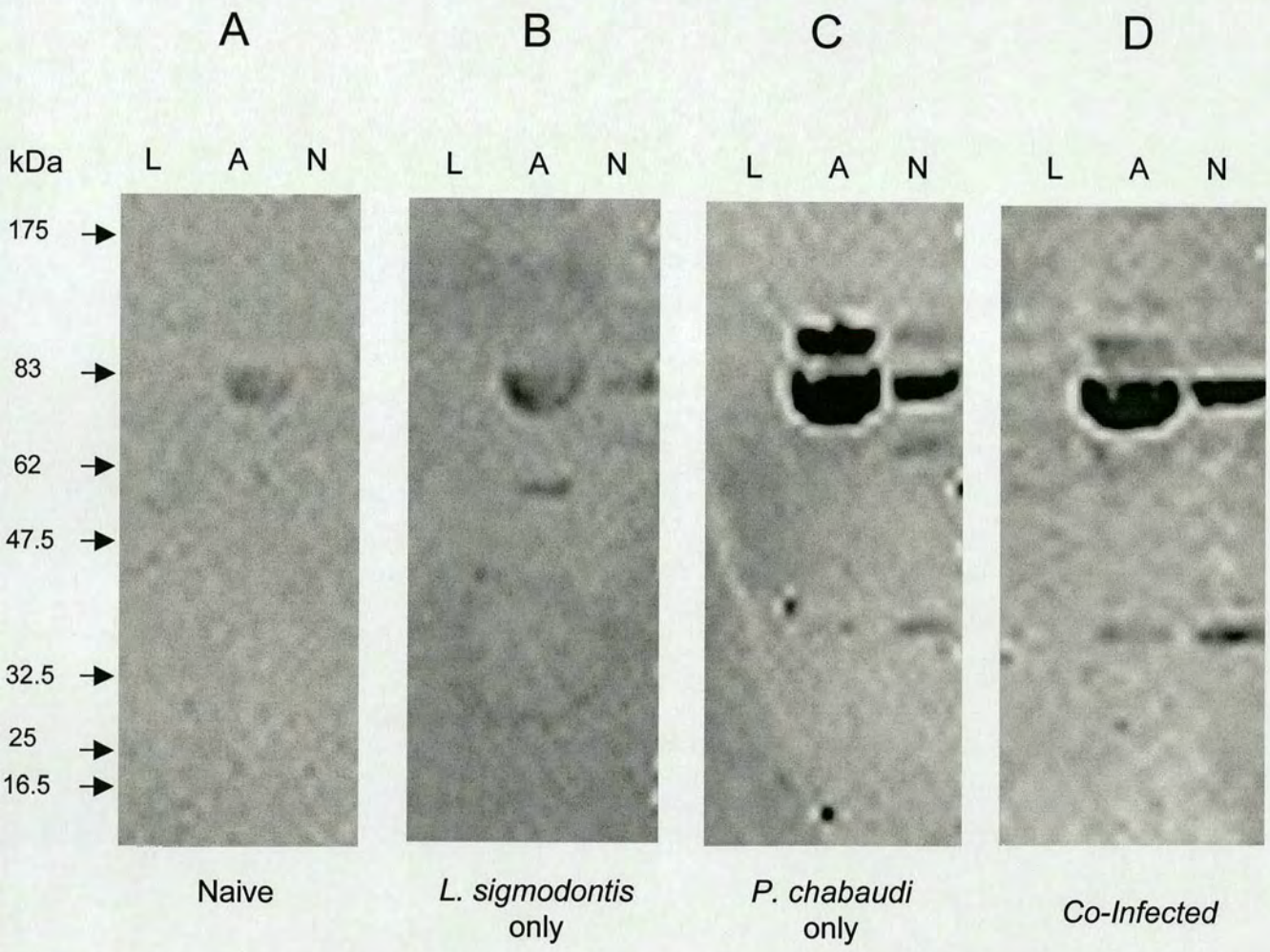


Figure 5:11. Western blot analysis of IgG2a antibody responses in mice infected with *L. sigmodontis*, *P. chabaudi* and animals co-infected with the two parasites. Each blot represents one animal from each group. A blot with sera from a naive animal is shown for comparison. Lanes marked L are loaded with *L. sigmodontis* adult antigen, lanes marked A are loaded with *P.chabaudi* infected red blood cell antigen and lanes marked N are lanes loaded with naive blood cell antigen as a control for red blood cell proteins.

appeared to be present in the naive control animal but at very low levels (Fig. 5:10A, lane 1).

The proteins recognised by IgG2a antibodies produced in the same singly and co-infected animals was also examined. Overall the number of proteins recognised by IgG2a antibodies were less than those recognised by IgG1 (Fig. 5:11). We did not observe any IgG2a antibodies that recognised *L. sigmodontis* antigens in any animal, although the ELISA results in Fig. 5:7A indicate that this antibody isotype is present at low levels. The animal tested that was singly infected with *P. chabaudi* reacted predominately against 2 bands of the *P. chabaudi* antigen preparation that were approximately 85 kDa and 100 kDa in size (Fig. 5:11C, lane 2). However these bands appear to be red blood cell antigens, rather then parasite antigens (Fig. 5:11C, lane 3). A very similar pattern was observed in the co-infected animal (Fig. 5:11D, lanes 2 and 3). The naive animal appeared to have IgG2a antibodies to the 85 kDa protein (Fig. 5:1A, lane 2). The animal singly infected with *L. sigmodontis* produced IgG2a antibodies towards the 85 kDa band in both the *P. chabaudi* and naive red blood cell preparations (Fig. 5:11B, lanes 2 and 3), and interestingly the IgG2a evoked by infection with *L. sigmodontis* also appeared to recognise a band of around 65 kDa that was specific to *P. chabaudi* (Fig. 5:11B, lane 2). Thus the low levels of IgG2a produced in filarial infection (Figs. 5:7A and 5:7B) appear to be non-specific and do not recognise filarial nematode proteins.

5:3 Discussion

Filarial nematodes invade and establish in humans alongside co-infecting pathogens such as malaria (Chadee *et al.*, 2003; Ghosh and Yadav, 1995; Ravindran *et al.*, 1998; Guderian *et al.*, 1991; Piessens *et al.*, 1983). In this study we have looked at the effects on established filarial nematode infection when the host becomes co-infected with malaria. We have also investigated the interactions that occur between the immune responses that are evoked by each of the pathogens in co-infection. We carried out two experiments and found little effect of co-infection on infection dynamics in one experiment (Figs. 5:4A and 5:4G), but an acceleration of filarial nematode death with an accompanying decrease in malaria parasite density in another (Figs. 5:4B and 5:4H). This difference could be due to a number of factors such as slight differences in the health status of the mice that were used, or underlying infections with other micro-organisms as the mice were not bred and maintained in Specific Pathogen Free (SPF) conditions. Nevertheless these results provided an excellent framework with which to compare and contrast anti-parasite immune responses and pathology to look for correlations that may give rise to the differences we observed in the infection dynamics between these two experiments.

Contrary to work with malaria and intestinal helminths in humans (Nacher *et al.*, 2002a; Nacher *et al.*, 2002b) the differences we observed in malaria-induced pathology were very slight, and not statistically significant. However studies in humans often investigate subjects who are malnourished and the experiments in this

study consisted of mice that could feed *ad libitum*. Since mal-nourishment (Chandra 1992), and other stresses not experienced by laboratory mice, can impair the immune system, and the immune system is likely to be mediate differences in parasite infection dynamics and direct immune–mediated pathology, it is possible that small differences observed in laboratory mice could translate into significant differences in the field.

Nevertheless numerous animal studies have observed differences in malaria-induced pathology (Yoshida *et al.*, 2000; Yan *et al.*, 1997; Helmby *et al.*, 1998*). Schistosoma mansoni* was found to protect mice from death due to *P. chabaudi* infection in one study despite a similar course of parasite infection (Yoshida *et al.*, 2000). In another study *S. mansoni* resulted in a greater %pRBC and anaemia (Helmby *et al.*, 1998). These studies used different strains of mice, and established *S. mansoni* infection with different numbers of cercariae. Nevertheless this story is not so dissimilar from the studies of Nacher *et al.* (2002a, 2002b) who found that in humans, intestinal helminths can protect from potentially fatal cerebral malaria, yet result in higher levels of infection induced anaemia (Nacher *et al.*, 2001a) and increased prevalence of circulating malaria gametocyte number (Nacher *et al.*, 2001b). The protective effect of concomitant *S. mansoni* infection seen by Yoshida *et al.* (2000) in laboratory mice has also been replicated by laboratory experiments with the filarial nematode *Brugia pahangi* and lethal *Plasmodium berghei* (Yan *et al.*, 1997).

The reason why we did not see any statistical difference in malaria-induced pathology could be due to a number of factors both experimental (e.g. different infection

protocols) or scientific (e.g. different parasite species). For example Yan *et al.* (1997) study gave multiple inoculations with irradiated larvae prior to challenge potentially generating a much stronger Th2 bias than we observe with primary *L. sigmodontis* infection. Further it is possible that *L. sigmodontis* does not induce responses that are as strong as *S. mansoni,* limiting the effects that will occur from this co-infection. The trend towards decreased malaria parasite number was seen in both the maximum pRBC reached in experiment 1 (Fig. 5:4E) and in maximum parasite density reached in experiment 2 (Fig. 5:4H). This result is consistent with the observations made by Yan *et al.* (1997) whereby filarial nematode infection with *B. pahangi* protected against *P. berghei* induced pathology.

We observed in these studies a trend towards accelerated adult nematode death in both experiments (Figs. 5:4C and 5:4D) but this was only significant in the second experiment (Figs. 5:4B and 5:4D). It is difficult to discuss the significance of this finding in relation to the other studies mentioned previously because none of these studies examined the helminth parasitology. Investigations into the establishment and survival of gut helminths in animals concomitantly infected with *Plasmodia* so far indicate different effects for different species of nematode. Experiments in chickens carried out by Juhl and Permin (2002) discovered that the chicken malaria *Plasmodium gallinaceum* reduced the establishment of infection with the gut nematode *Ascaridia galli*. On the other hand *P. berghei* infection has been found to prolong infection with *Nippostrongylus brasiliensis* in mice (Modric and Mayberry 1974).

The observed difference in the pattern of recovery from singly and co-infected animals may be linked to differences in immuno-responsiveness between the two experiments. Generally mice in the second experiment had approximately four-fold more cells secreting IL4 in response to the non-specific stimulus Con A than in the first experiment (Fig. 5:6). This could be due to the differences in the quality of cell preparations or ELISPOT assay differences. However there was little difference in the number of Con A-induced IFNγ secreting cells between the two experiments suggesting that cell preparation quality and assay differences were not entirely responsible for such a large increase in type 2 responsiveness in the second experiment. Thus effects on parasite number appear to correlate with a greater potential to mount type-2 immune responses. This indicates that stronger immune responses may be more likely to affect infection dynamics in co-infected animals. The co-infected animals appeared to be less skewed towards the type 2 axis (represented by IL4 secreting cells) than animals singly infected with *L. sigmodontis* in both experiments. This apparent difference in skew in response to Con A appeared to be driven by additional IFNγ secreting cells rather than by a decrease in IL4 secreting cells (Fig. 5:6). This was not surprising considering *P. chabaudi* initiates a type 1 response in the early part of infection (Langhorne and Simon, 1989; Langhorne *et al.*, 1989). This data illustrates that type 1 responses can be initiated that can skew a response that is strongly type 2 biased, and has been established for 60 days. It also highlights the fact that immunological interactions do occur in co-infection

Surprisingly responses to Con A in mice singly infected with *P. chabaudi* were not strongly skewed towards the type 1 axis (represented by IFNγ secreting cells) as might be expected from this type 1 inducing pathogen (Fig. 5:6). This may be because after initially evoking primarily a type 1 skewed response early post-infection, *P. chabaudi* evokes a mix of type 1 and type 2 associated cytokines by day 20 post-infection (Stevenson and Tam, 1993; von der Weid *et al.*, 1994).

Evidence that immune responses in co-infected animals interact was also obtained by looking at the isotypes of antibodies produced in co-infected animals. Unlike Con A induced splenocytes responses, IgG1 and IgG2a responses mounted against each pathogen were more dissociated. Anti-*L. sigmodontis* antibody responses were dominated by type 2 IgG1 in both singly- infected and co-infected groups of animals in both experiments (Figs 5:7A and 5:7C). There was an additional anti-*L. sigmodontis* IgG2a response in the first experiment although the level of IgG2a was not above that of cross-reactive antibody measured in animals singly infected with *P. chabaudi* (Fig. 5:7A). This IgG2a response could be due to cross-reactivity rather than skewing of the immune response. More convincing evidence of a skewing of the immune response away from type 2 by co-infecting *P. chabaudi* parasites is the circulating polyclonal IgE level measured in the first experiment. IgE production was driven largely by *L. sigmodontis* infection, and appeared to be lower in co-infected animals (Fig. 5:8).

Anti-*P. chabaudi* antibody responses in animals singly infected with *P. chabaudi* were dominated by type 1 associated IgG2a rather than type 2 associated IgG1 (Figs. 5:7B

and 5:7D). On the other hand co-infected animals produced both anti –*P. chabaudi* IgG2a and IgG1 (Figs. 5:7B and 5:7D). In both cases the level of anti-*P. chabaudi* IgG1 measured was not above the level of cross-reactive antibody level observed in animals singly infected with *L. sigmodontis*. Therefore, again, this observation could be due to cross-reactivity rather than a skew of immune response *per se*.

We observed a high level of variation of anti-filarial antibody responses within both the group of animals singly infected with *L. sigmodontis* and in the co-infected animals despite identical infection protocols. One reason for this could be due to a phenomenon of *L. sigmodontis* infection in BALB/c mice whereby only around 50% of the infected mice become Mf+ (Petit *et al.*, 1992). Because Mf can be inducers of type 1 responses (Lawrence *et al.*, 1994) and because circulating Mf share the same compartment as malaria parasites (the blood), we hypothesised that there could be interactions between malaria and Mf. In animals that were Mf+ we observed slightly higher, but non-significant, type 1 anti-filarial immune responses (Figs. 5:9C and 5:9D), but no difference in type 2 responses (Figs. 5:9A and 5:9B). There was no difference in this pattern between mice that were singly infected or co-infected indicating that any interactions between malaria and Mf may not be strong enough to influence the anti-filarial immune response.

Analyses of parasite-specific antibody responses revealed that animals singly infected with *L. sigmodontis* had both IgG1 and IgG2a antibody responses to malaria (Figs. 5:7B and 5:7D). Similarly animals singly infected with *P. chabaudi* had antibody

responses to *L. sigmodontis*, although these were of a smaller magnitude (Figs. 5:7A and 5:7C). The production of cross-reactive antibodies in humans infected with malaria and filarial nematodes has been described previously (Harrison and Ridley, 1975; Naus *et al.*, 2003). Here we were able to show that filarial nematode infection induces IgG1 and IgG2a responses that cross-react with *P. chabaudi* proteins (Figs. 5:10B and 5:11B, both lane 2). These cross-reactive IgG1 responses were maintained in co-infection, whilst in comparison anti-IgG1 responses that are specific for *L. sigmodontis* appear to decline (Fig. 5:10D, lanes 2 and 3). This indicates that the anti-*L. sigmodontis* type 2 response may be down regulated in co-infection as the animal singly infected with *L. sigmodontis* produced similar levels of IgG1 against both pathogens in the absence of *P. chabaudi*.

A possible explanation for this data is that IgG1 antibodies cross-reactive with *P. chabaudi* are produced from a different B cell population from those that are producing antibodies specific for *L. sigmodontis*. B1 cells that are polyclonally stimulated are an important part of the innate response (Martin and Kearney, 2001). They may therefore be less susceptible to skews in the acquired immune response and account for the maintenance of cross-reactive IgG1 antibody responses. On the other hand *L. sigmodontis* specific IgG1 may be produced in a T-dependent fashion from B2 cells. The down-regulation of anti-*L. sigmodontis* specific IgG1 may therefore be a reflection of a down-regulation of the anti-filarial type 2 response in this animal. In support of this hypothesis IgG1 antibodies that reacted against self-RBC proteins were also maintained in co-infected animals (Fig. 5:10D, lane 3). Further the IgG2a

response produced in *L. sigmodontis* infection did not react with *L. sigmodontis* infection and largely consisted of cross-reactive antibodies to *P. chabaudi* and a self-RBC protein.

The production of auto-antibodies has been attributed to B1 cells in many different models (for review see Fagarasan *et al.*, 2000). Therefore it may be possible that B1 cells are responsible for producing cross-reactive IgG1 antibodies that react with *P. chabaudi* and antigens in naive red blood cells, and they are unaffected by changes in skew in the acquired immune response. Further support for this model is obtained by looking at blots with the naive animal, which appears to have natural IgG1 and IgG2a antibodies against some *L. sigmodontis* proteins, and a protein found in *P. chabaudi* infected RBC. (Fig. 5:10A, lane 1 and Fig 5:11B, lane 2). B1 cells are an important source of natural antibody (Martin and Kearney 2001) and IgG1 and IgG2a isotypes can form part of the natural antibody repertoire in mice (Malanchere *et al.*, 1995). This hypothesis could be tested by examining the pattern of these antibody responses in co-infected Xid mice that do not have any B1 cells (Khan *et al.*, 1995).

In summary we have shown that the immune responses mounted by co-infecting malaria parasites can have effects on the immune responses established by filarial nematodes. An acceleration in the death of filarial nematodes was associated with a strong non-specific type 2 cellular immune response in the spleen Further, data in this study indicated that malaria infection can interfere with a well-established anti-filarial type 2 immune response in the space of a very short time. The results from this study

highlight the importance of studying the interactions that occur between the immune responses evoked by co-infecting organisms and merit further investigation.

CHAPTER 6

(including discussion of data included in the Appendix)

Compartmentalisation of co-infecting pathogens in separate parts of the body does not necessarily confer independence of immune responses

6:1 Introduction

Co-infection with several un-related pathogens simultaneously is a common phenomenon, particularly in developing countries (Buck *et al.*, 1978; Keusch and Migasena 1982; Chunge *et al.,* 1991; Yu *et al.*, 1994). In co-infected individuals unrelated pathogens are likely to occupy different niches in the body and the immune responses generated against individual pathogens must be directed to the correct site of infection. Additionally, the immune system will need to be able to mount different kinds of responses against unrelated pathogens simultaneously. In the case of co-infection with helminths that evoke type 2 responses, and bacterial or protozoal infections that evoke type 1 responses, the immune system would have to mount effector mechanisms that are associated with both types of immune response.

The immune system should be capable of mounting independent primary anti-pathogen immune responses because co-infections with unrelated pathogens are likely to evoke immune responses in distinct lymph nodes draining different compartments. Effector cells should be able to traffic to the correct lymph nodes through the induction of a variety of chemoattractants, such as chemokines (Olson and Ley 2002), generated at the site of infection. Additionally antigen specific cells control each

response and it has previously been demonstrated that type 1 and type 2 antigen specific immune responses can occur simultaneously and independently (Ismail and Bretscher 1999).

Although it can be hypothesised that simultaneous immune response should be able to occur independently, the immune response generated by pathogens, in particular helminths, has been shown to be strong enough to influence the development of subsequent immune responses (Pearlman *et al.*, 1993; Curry *et al.*, 1995; Rodriguez *et al.*, 1999; Yoshida *et al.*, 1999; Cooper *et al.*, 2001). It is possible that this occurs because not all facets of an adaptive immune response are antigen specific. Cytokines, for example, are promiscuous and may influence immune cells specific to an incoming second infection. Interleukin 4 (IL4), the dominant cytokine produced in a type 2 response, and IFNγ, the signature cytokine of a type 1 response, are considered to be cross regulatory in nature (Abbas *et al.*, 1996). It follows that if an individual has a pre-existing anti-helminth IL4 response, the amount of IFNγ the immune system of this individual can generate to an incoming protozoal or bacterial infection could be limited due to the presence of IL4. Further, interactions between simultaneous anti-pathogen immune responses in co-infection may be may be more likely if the infections occur in close proximity to each other in the body and share draining lymph nodes (Curry *et al.*, 1995).

We wanted to test the hypothesis that compartmentalisation of different pathogens within the body would allow the immune system to generate effective independent

immune responses against each pathogen. We have used the rodent filarial nematode model *Litomosoides sigmodontis* (Hoffman *et al.*, 2000) and a sub-cutaneous infection of the protozoan parasite *Leishmania major* (Sacks and Noben-Trauth 2002). After establishing infection with *L. sigmodontis* in the thoracic cavity, we challenged the footpad with *L. major* (Fig. 6:1). C57BL/6 mice are resistant to both these infections. This resistance is dependent on IL4 for *L. sigmodontis* (Le Goff *et al.*, 2002) and IFNγ for *L. major* (Wang *et al.*, 1994a). The presence of opposing immune responses in this model of co-infection, and the cross-regulatory nature of these immune responses, will optimise the detection of interactions in the immune system. Further by examining whether co-infection reverses, or changes, host resistance the consequences of interactions between the immune responses mounted against each pathogen in this co-infection model can be assessed. By setting up *L. major* co-infection in the thoracic lymph nodes, rather than the footpad, we were also able to compare the effect of co-infection on anti-parasite immune responses and host resistance when *L. major* co-infection was essentially at the same site and not compartmentalised away from *L. sigmodontis* infection.

In this model of co-infection we were able to see interactions between the immune responses to the two different pathogens in the draining lymph nodes of each pathogen compartment. This suggests that compartmentalisation is not sufficient to generate completely independent anti-pathogen immune responses. This immunological interaction appeared to be beneficial for the host as it delayed the formation of footpad

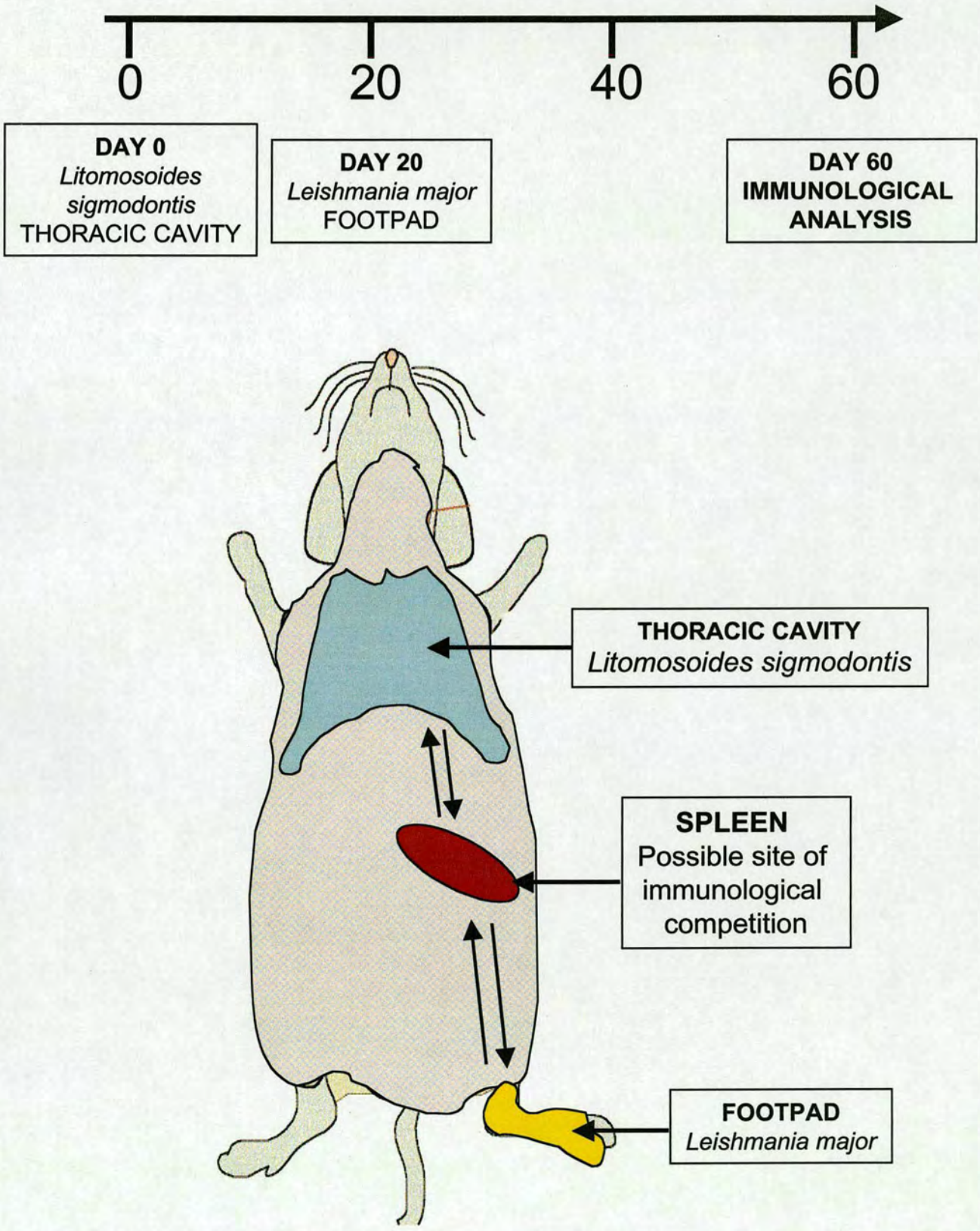


Figure 6:1. *L. sigmodontis* and *L. major* co-infection protocol. 20 days post-infection with *L. sigmodontis*, C57BL/6 mice were infected with *L. major* in the footpad. 40 days post-infection with *L. major*, the local draining lymph nodes from each site of infection, in addition to the spleen, were analysed for anti-parasite responses.

lesions induced by *L. major* and also appeared to accelerate the death of adult filarial nematodes.

6:2 Results

6:2:1 Compartmentalised co-infection altered the number of cells reacting against each parasite in each compartment.

We co-infected C57BL/6 mice with *L. sigmodontis* and *L. major* according to the protocol described in Figure 6:1. At 40 days post *L. major* infection, when lesion development begins to heal (herein described as day 40 PI) (Matthews *et al.*, 2000), we examined recall responses in the thoracic lymph nodes draining the nematode compartment (thoracic cavity) and the popliteal lymph nodes draining the *L. major* compartment (footpad). We repeated this experiment and meta-analysed the data from both experiments to test for differences in the immune responses mounted by singly and co-infected animals. Logarithmic, square root or inverse transformations were used to transform the data before analysis.

We discovered that infection with *L. major* altered the immune response mounted against *L. sigmodontis* in co-infected animals when compared with animals singly infected with filarial nematodes. There was a significant decrease in the number of cells secreting IL4 against *L. sigmodontis* in the thoracic lymph nodes of co-infected animals, compared with animals singly infected with *L. sigmodontis* (ANOVA $P=0.019$) (Fig. 6:2A). Few cells secreting IFNγ were detected in the thoracic lymph

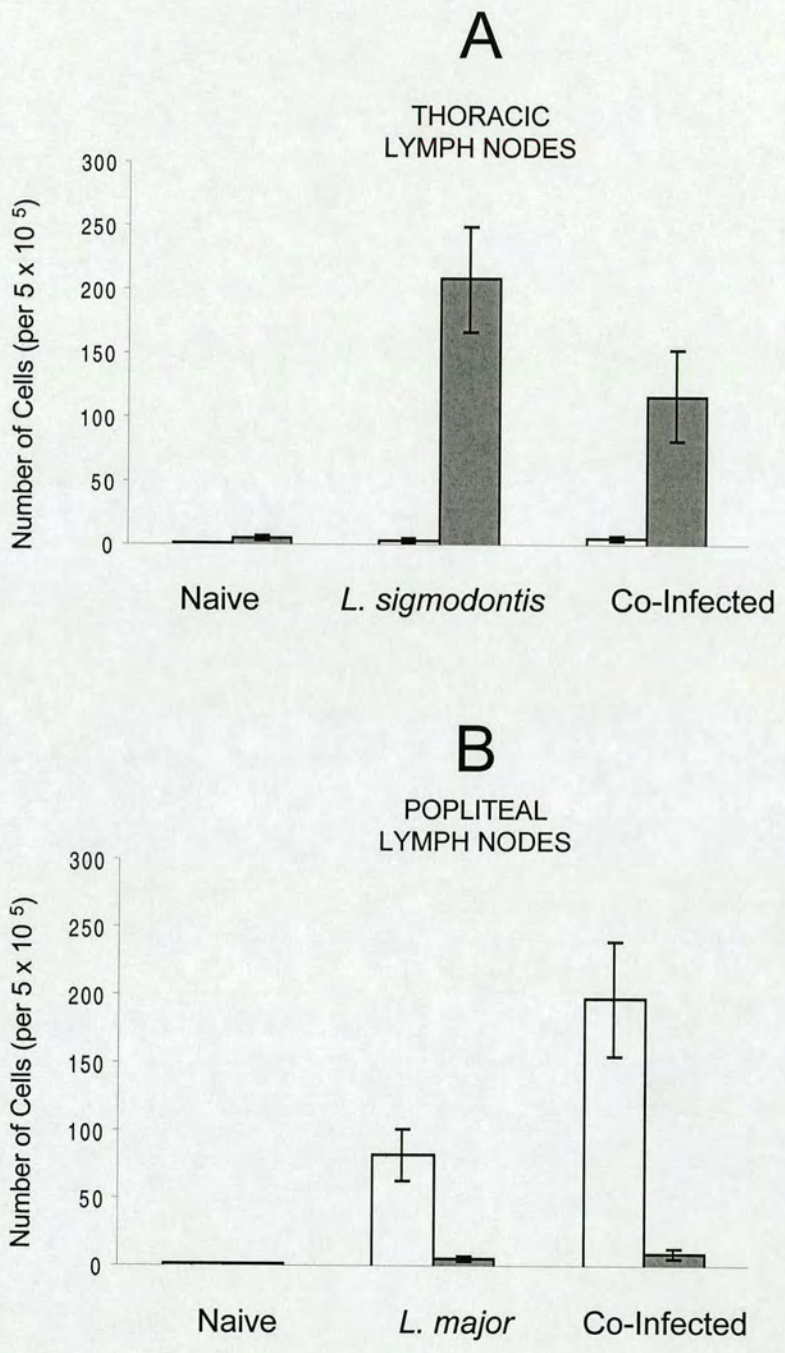


Figure 6:2. Immune responses to *L. major* and *L. sigmodontis* in compartmentalised co-infection at day 40 post-infection with *L. major*. The number of cells secreting IFNγ (white bars) and IL4 (grey bars) against *L. sigmodontis* in the thoracic lymph nodes (A) and against *L. major* in the popliteal lymph nodes (B) are shown. 3 x 10^5 thoracic or popliteal lymph node cells were assayed although these results were multiplied to graphically represent 5 x 10^5 lymph node cells. The graphs show the pooled values of 2 different experiments and the error bars show the SEM. The responses of naive animals are shown for comparison but were not included in any of the analyses.

nodes of either co-infected animals or animals singly infected with *L. sigmodontis* (Fig. 6:2A). Likewise, a pre-existing filarial infection altered the immune response mounted against *L. major* in co-infected animals when compared with animals singly infected with *L. major*. In the popliteal lymph nodes we observed a trend towards an increased number of cells secreting IFNγ in response to *L. major* in co-infected animals compared with animals singly infected with *L. major* (Fig. 6:2B), although this increase was not significant (ANOVA P=0.06). No cells secreting IL4 in response to *L. major* were observed (Fig. 6:2B). Thus in both pathogen compartments, the anti-pathogen immune response measured was altered in magnitude in comparison with singly infected animals. However the immune response was still highly polarised towards type 2 in the nematode compartment and towards type 1 in the *L. major* compartment.

6:2:2 Compartmentalised co-infection altered the number of cells reacting against each parasite in the spleen.

Since anti-parasite splenic immune responses can be measured in both murine *L. sigmodontis* infection and murine *L. major* infection, we hypothesised that the spleen could be a possible site of immunological competition. Perhaps not surprisingly, the immune responses were less distinct in the spleen as compared to the draining lymph nodes. Co-infected animals appeared to have less IL4 secreting anti-*L. sigmodontis* specific cells compared with animals singly infected with *L. sigmodontis*, and more IFNγ secreting anti-*L. major* specific cells compared with animals singly infected with *L. major* (Figs. 6:3A and 6:3B respectively). Although these trends were not

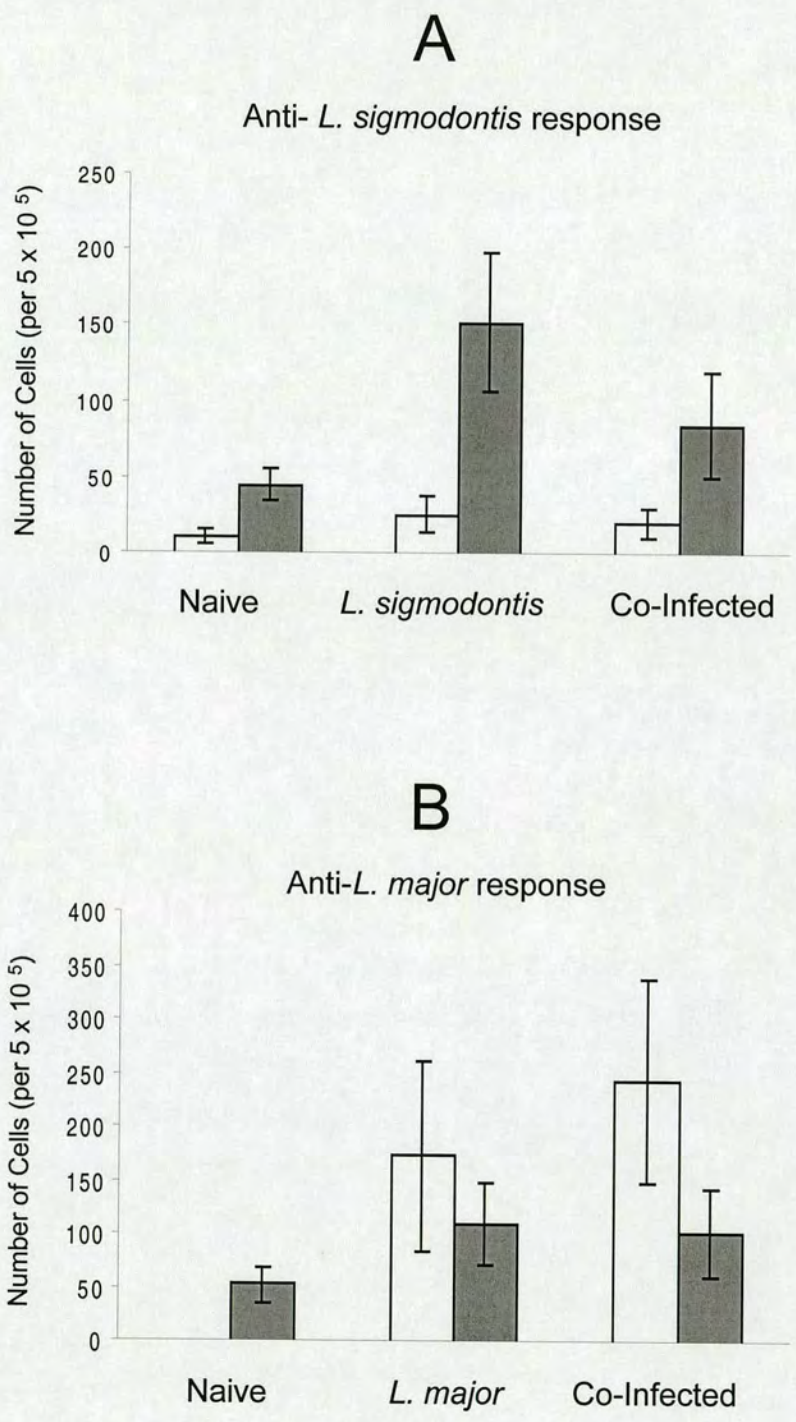


Figure 6:3.Splenic immune responses to *L. major* and *L. sigmodontis* in compartmentalised co-infection at day 40 post-infection with *L. major.* The number of cells secreting IFNγ (white bars) and IL4 (grey bars) against *L. sigmodontis* (A) and *L. major* (B) are shown. The graphs represent the pooled values of 2 different experiments and the error bars represent the SEM. The responses of naive animals are shown for comparison but were not included in any of the analyses.

significant (ANOVA P>0.05 in both cases), these responses matched the pattern observed in the thoracic cavity for IL4 secreting cells specific for *L. sigmodontis* (Fig. 6:2A) and in the popliteal lymph nodes for IFNγ secreting cells specific for *L. major* (Fig. 6:2B).

*6:2:3 Infection in the same site down-regulates the established anti-*L. sigmodontis *immune response.*

To more fully interpret the effects of physical compartmentalisation of co-infecting pathogens, we chose to examine the anti-parasite immune responses when the site of infection for the same two pathogens drained into the same lymph nodes (same-site infection). The thoracic lymph nodes that drain the thoracic cavity where *L. sigmodontis* resides, also drain the peritoneal cavity in rodents (Tilney 1971). By injecting *L. major* intra-peritoneally (ip.) we were able to culture *L. major* parasites directly from the thoracic lymph nodes of 100% of mice injected in this way at 14 days post-infection (data not shown). Thus co-infection experiments in which *L. major* was injected ip. would lead to the initiation of an anti-*L. major* response in the same lymph nodes where an anti- *L. sigmodontis* immune response was already established. Again 20 days after establishing infection with *L. sigmodontis*, we challenged with *L. major* infection and immunological parameters were measured 40 days later (40 days PI). The popliteal lymph nodes as the shared lymphatic compartment was not chosen because *L. sigmodontis* larvae injected subcutaneously into the footpad migrate rapidly to the thoracic cavity and the anti-*L. sigmodontis* immune response is not maintained in the popliteal lymph nodes (L. Le Goff, unpublished data).

As observed in compartmentalised co-infection, the anti-*L. sigmodontis* response was strongly polarised and only cells secreting IL4 in response to *L. sigmodontis* could be detected (Fig 6:4A). This response appeared to be drastically decreased in co-infected animals compared with animals singly infected with *L. sigmodontis* (Fig. 6:4A). However this observed decrease in response was not significant (Mann-Whitney U-test $P>0.05$) because data from only two animals singly infected with *L. sigmodontis* were obtained for the thoracic lymph nodes in this experiment, and only one of these animals responded to *L. sigmodontis* (Fig 6:4A). Nevertheless none out of four of the co-infected animals secreted IL4 in response to *L. sigmodontis* in this experiment. Compared with compartmentalised co-infection at this time point (Fig. 6:4B), same-site co-infected animals may have a greater affect on decreasing the anti-*L. sigmodontis* response (Fig. 6:4A).

The anti- *L. major* response in animals singly infected with *L. major* ip. appears to be weaker in the thoracic lymph nodes when compared with the popliteal lymph nodes upon an *L. major* sub-cutaneous footpad infection (Figs. 6:4C and 6:4D). Nevertheless the anti- *L. major* response consisted predominantly of cells secreting IFNγ in both locations (Figs. 6:4C and 6:4D. There was no statistical difference between the number of cells secreting IFNγ in animals singly infected with *L. major* and co-infected animals (Mann Whitney U-test $P>0.05$) (Fig. 6:4C). However co-infected animals had more cells secreting IL4 than animals singly-infected with *L. major* (Mann Whitney U-test $P=0.015$), although the number of IL4 secreting cells detected

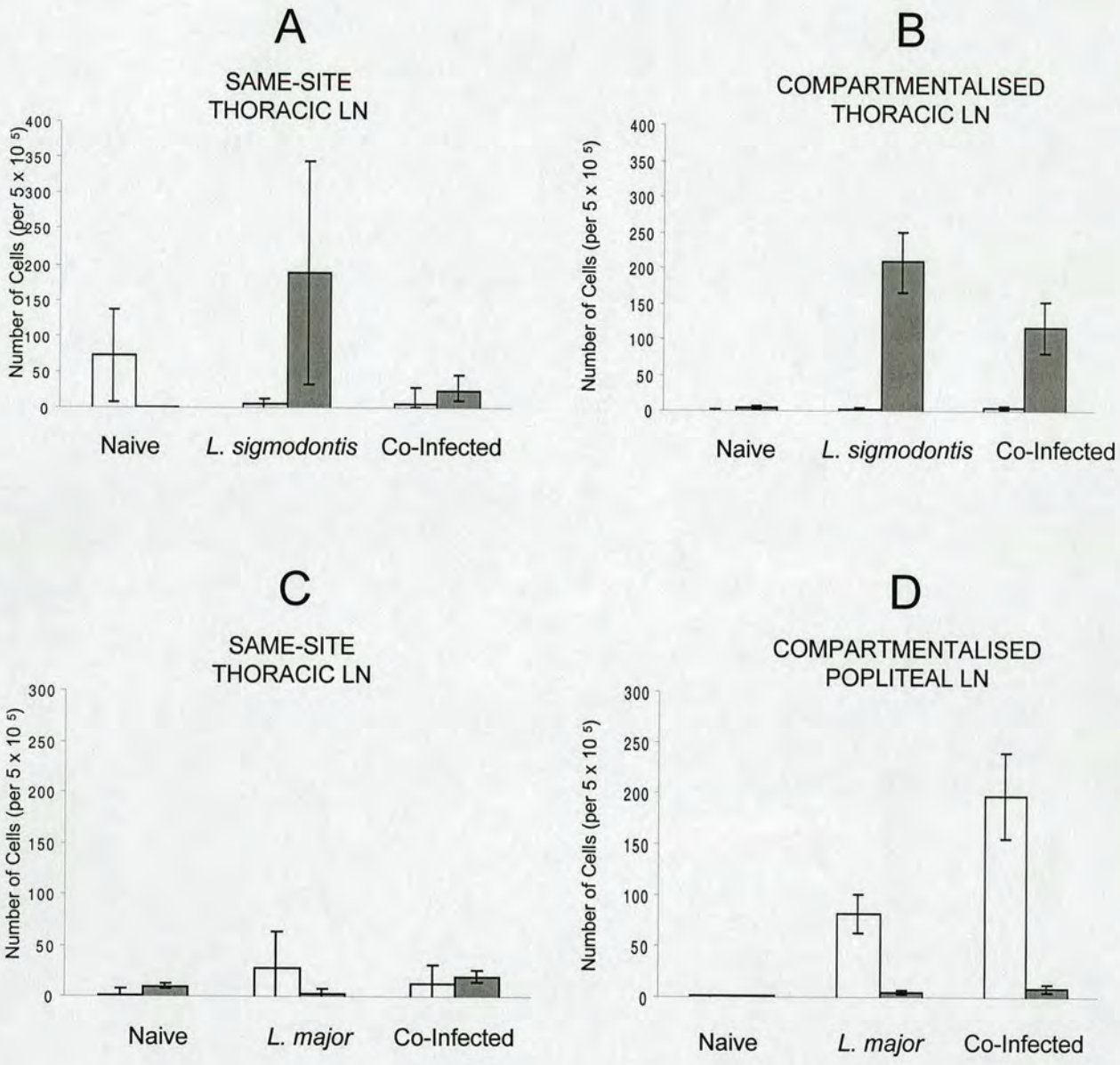


Figure 6:4. Immune responses to *L. major* and *L. sigmodontis* in same-site co- infection at 40 days post - *L. major* infection. Same-site co-infection is shown in A and C This data is compared with compartmentalised co-infection in B and D (duplicated from Figs. 6:2 and 6:3). The number of cells secreting IFNγ are represented by the white bars and the number of cells secreting IL4 are represented by grey bars. Cells responding against *L. sigmodontis* in the thoracic lymph nodes are shown in A and B. Cells responding against *L. major* in the thoracic lymph nodes and popliteal lymph nodes are shown in C and D respectively. 3 x 10^5 cells were assayed and these results were multiplied to graphically represent 5 x 10^5 lymph node cells. The error bars represent the maximum and minimum values for each group and cytokine (A and C) or the SEM of data pooled from two experiments (B and D). Naive animals are shown for comparison but were not included in any of the analyses. LN - lymph nodes.

in co-infected animals was not different from naïve animals (Mann Whitney-U test P>0.05) (Fig. 6:4C). Nevertheless these findings contrast with compartmentalised co-infection where a non-significant trend towards an increase in the number of cells secreting IFNγ in response to *L. major* in co-infected animals compared with animals singly infected with *L. major* was observed (ANOVA P=0.06) (Fig. 6:4D).

Although only weak anti-*L. major* responses were observed in the thoracic lymph nodes of mice singly infected with *L. major* ip. (Fig. 6:4C), the drastic decrease in IL4 secreting anti-*L. sigmodontis* cells in animals co-infected in the same-site when compared with compartmentalised co-infection (Fig 6:4A), indicate that infection with *L. major* ip. was successful. However this route of *L. major* infection may not be as efficient as injecting *L. major* subcutaneously in the footpad.

6:2:4 Immunological interactions between the immune response mounted against each parasite accelerated the death of L. sigmodontis *adult nematodes and delayed* L. major *induced pathology.*

We hypothesised that the observed changes in anti-parasite immune responses may impair the ability of the immune system to defend the host against parasite establishment and survival, and additionally alter parasite-induced pathology. As a measure of pathology we recorded footpad inflammation induced by *L. major* infection throughout the course of compartmentalised co-infection and compared this to the *L. major* induced footpad pathology in animals singly infected with *L. major*. We observed a significant delay in the development of footpad lesions in co-infected

mice when compared with mice singly infected with *L. major* (Fig. 6:5). Two weeks post-infection with *L. major* the co-infected animals had smaller footpad swellings than the mice singly infected with *L. major* (ANOVA, $P<0.01$) (Fig. 6:5). By the end of these experiments terminated at day 40 PI the co-infected animals appeared to have larger lesions (Fig. 6:5) although this observation was not significant (ANOVA $P>0.05$). Co-infected mice had less overall pathology compared with mice singly infected with *L. major* as measured by the average area under the curve for each group of mice (2.675 mm^2-weeks for singly infected mice and 2.182 mm^2-weeks for co-infected mice, ANOVA $P=0.042$) (Fig. 6:5). Therefore pre-existing *L. sigmodontis* infection protected the mice from full development of *L. major* induced pathology.

We assessed the impact of the altered anti-*L. sigmodontis* immune response observed in mice with compartmentalised co-infection on the survival of filarial nematodes. The percentage of mice that harboured live adult *L. sigmodontis* parasites was recorded for all experiments and found to be between 20 and 80% (Fig. 6:6). This is higher than expected for resistant mouse strains on the C57 background (Petit *et al.*, 1992) and for this mouse strain as observed in our laboratory (Le Goff *et al.*, 2002). However in every case, the % of mice with live parasites was less in the co-infected group of animals. This happened as early as 14 days post-co-infection with *L. major* (Fig. 6:6A) when co-infected animals had reduced *L. major* induced footpad pathology (Fig. 6:5) and also occurred with same-site co-infection (Fig. 6:6B). Due to the small numbers of animals used in each experimental group none of the observed differences in the % of

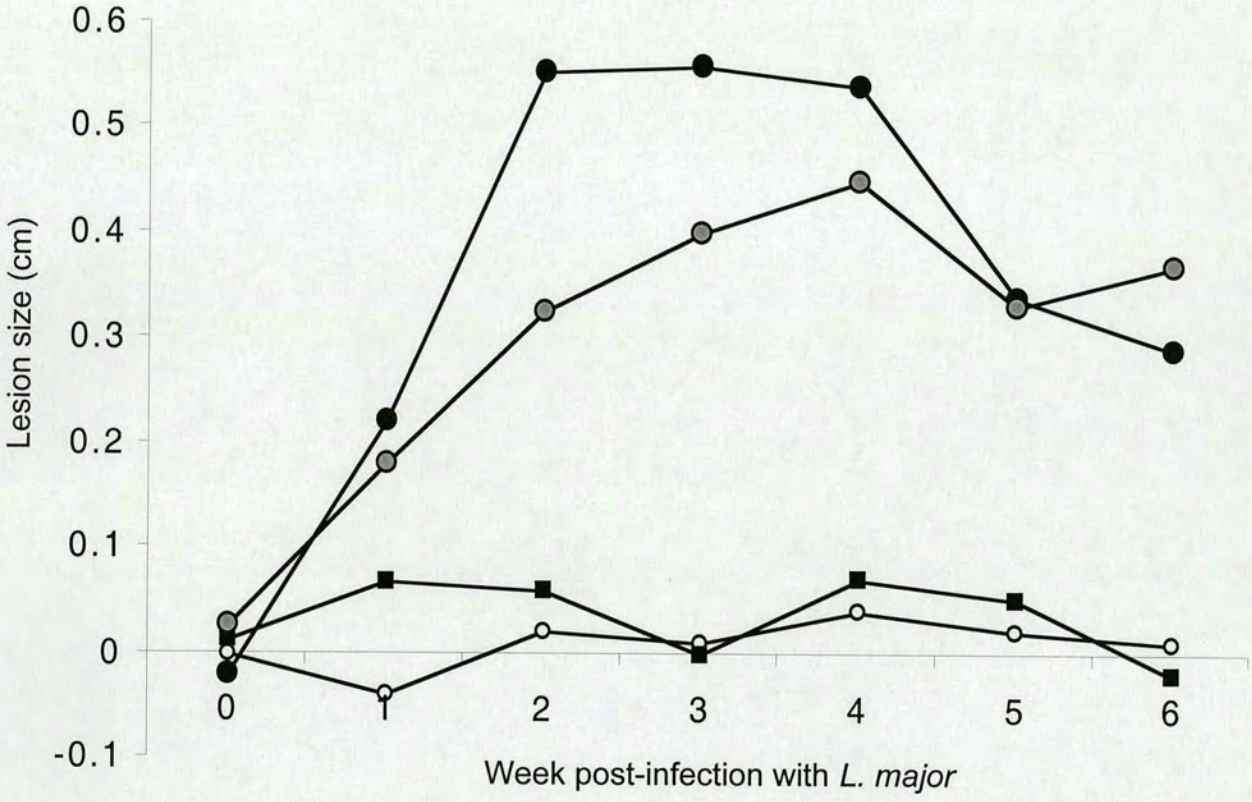


Figure 6:5 The pathology induced by *L. major* is delayed in mice co-infected with *L. sigmodontis*. The size of the control right footpad was subtracted from the size of the left infected footpad for each mouse. The open circles represent the naive mice, the black circles represent the animals infected with 3 x 10^6 *L. Major* stationary promastigotes and the grey circles represent the co-infected animals. The footpads of the animals singly infected with *L. sigmodontis* are shown by the black squares and are shown as an additional control. Each symbol represents the mean value for each group at each time point. Error bars have been omitted for clarity.

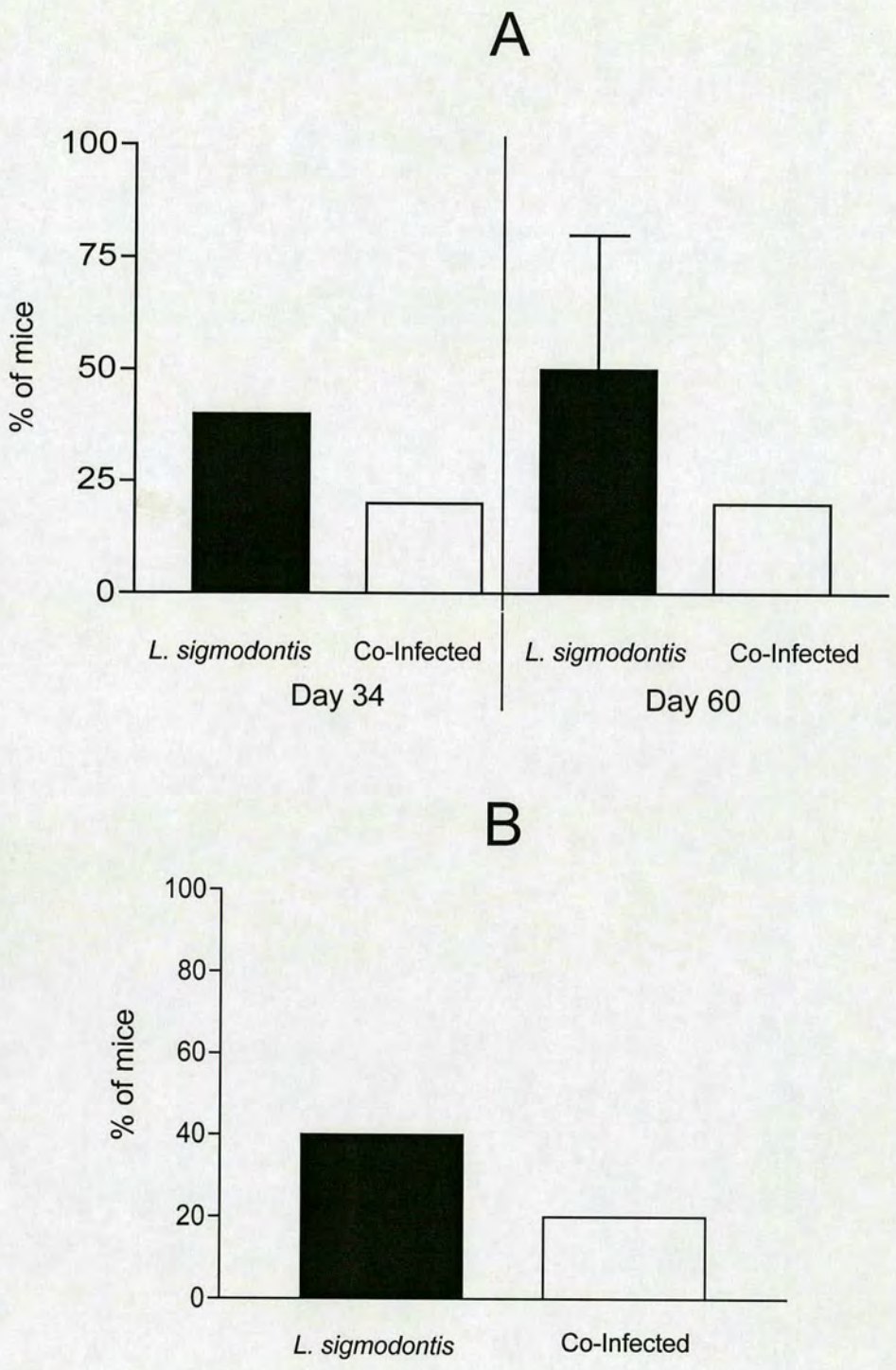


Figure 6:6. Co-infection accelerates the death of adult *L. sigmodontis* parasites. The percentage of mice with live adult nematodes at the end of compartmentalised co-infection is shown at days 34 and 60 post - nematode infection (A). The values obtained from two different experiments are shown for 60 days post-infection and the error bars represent the maximum and minimum values for each group of mice. Figure B shows the % of mice with live *L. sigmodontis* adult nematodes at day 60 post-nematode infection in same-site co-infection.

infected mice described above reached statistical significance (Logistic regression all P>0.05 in all cases). Average parasite recovery rates for each group were not compared due to the small number of adult parasites that were recovered in all cases (data not shown). This data indicates that the immunological interactions occurring in co-infected animals is correlated an increased ability of the immune system to defend the host against *L. sigmodontis* infection.

6:3 Discussion

Co-infection is the rule rather than the exception in infectious disease. We hypothesised that the immune system should be able to mount independent responses against co-infecting pathogens that inhabit distinct sites in the body of the host. To test this hypothesis we co-infected mice with the murine filarial nematode model *L. sigmodontis* that resides in the thoracic cavity, and the protozoan parasite *L. major* that resides in the footpad. Interestingly we found that, although distinct antigen-specific and appropriately polarised responses occurred in the draining lymph nodes, compartmentalisation of these pathogens within the body did not confer full independence of these anti-pathogen immune responses.

Unfortunately we observed a large variation in all immune responses measured which reduced the power to detect statistical differences. It is possible that some of this variation was driven by the infection status of the mice before the start of these experiments. All mice came from colonies with endemic *Tritrichomonas muris*

infection, which is known to interfere with immune responses to *L. major* (I. Muller, personal communication). Additionally there was a possibility that some of the mice used in these experiments may have had infection with pinworms (*Syphacia muris*).

Using ELISPOT we were able to measure the number of cells secreting the type 1 signature cytokine IFNγ, a hallmark of the immune response mounted against *L. major* in C57BL/6 mice and the type 2 cytokine IL4, a hallmark of the immune response mounted against *L. sigmodontis*. In compartmentalised co-infection the cells of the thoracic lymph nodes secreted IL4, but not IFNγ, in response to *L. sigmodontis* in both animals singly infected with *L. sigmodontis* and co-infected animals (Fig. 6:2A). Likewise in the popliteal lymph nodes cells from both animals singly infected with *L. major* and co-infected animals secreted IFNγ, and very little IL4, in response to *L. major* (Fig. 6:2B). Therefore the immune responses in co-infected animals were still strongly polarised towards type 2 in the thoracic lymph nodes and type 1 in the popliteal lymph nodes, demonstrating that compartmentalisation of the pathogens within the body did lead to a certain amount of compartmentalisation in the pathogen specific immune responses.

Nevertheless these immune responses were not fully independent and differed in magnitude in co-infected animals compared with singly infected animals. The number of cells secreting IL4 in response to *L. sigmodontis* in the thoracic lymph nodes was significantly decreased in co-infected animals compared with animals singly infected with *L. sigmodontis* at day 40 PI (Fig. 6:2A). This decrease in the number of cells

secreting IL4 was not partnered with an increase in the number of cells secreting IFNγ in the thoracic lymph nodes in response to *L. sigmodontis*, as might be expected if the decrease was due to cross-regulation between the type 2 anti-*L. sigmodontis* response and IFNγ emanating from the anti-*L. major* type 1 immune response in the popliteal lymph nodes (Fig. 6:2B). The type 2 polarisation of the immune response in the thoracic lymph nodes is also demonstrated by *ex vivo* immune responses of thoracic lymph node cells (Fig. Ap:1B) and stimulation of thoracic lymph node cells by the T cell mitogen concanavalin A (Con A) (Janeway and Travers, 1996) (Fig. Ap:2B) from co-infected animals and animals singly infected with *L. sigmodontis*. Under both conditions the majority of cells secrete IL4 and only a few cells secrete IFNγ in both groups of animals.

In the popliteal lymph nodes there appeared to be an increase in the number of cells secreting IFNγ in response to *L. major* in co-infected animals compared with animals singly infected with *L. major* at day 40 PI (Fig. 6:2B), although this increase did not reach statistical significance (ANOVA P=0.06). We believe that this trend is not an artefact because there were significant increases in the number of popliteal lymph node cells spontaneously secreting IFNγ (ANOVA P=0.015) (Fig. Ap:1C) and also in response to Con A (ANOVA P=0.01) (Fig. Ap:2C) in co-infected animals compared with animals singly infected with *L. major*. Therefore it seems that the pre-existing *L. sigmodontis* type 2 response resulted in an increased type 1 anti-*L. major* immune response at 40 days PI. Again this result is not what would be expected if pre-existing IL4 generated by *L. sigmodontis* infection was cross-regulating the anti-*L. major* type

1 response. However IL4 has been shown to be capable of promoting IL12 production from monocytes (Bullens *et al.*, 2001) and monocyte-derived dendritic cells (Pawe *et al.*, 2000) during their interaction with T cells. IL12 is a key cytokine involved in the initiation of the production of type 1 cytokines such as IFNγ (Manetti *et al.*, 1993; Seder *et al.*, 1993). Therefore it is possible that the presence of IL4 from the anti-*L. sigmodontis* response could result in the observed enhanced anti-*L. major* IFNγ response in the popliteal lymph nodes by promoting IL12 production.

We wanted to compare the observed anti-parasite immunological interactions in compartmentalised co-infection to the scenario where parasites occupy a similar niche within the body. We initiated infection with *L. major* in the peritoneal cavity because this location, like the thoracic cavity, is drained by the thoracic lymph nodes (Tilney 1971). Initiation of *L. major* infection by this route did not induce strong anti-*L. major* immune responses in the thoracic lymph nodes (Fig. 6:4C). Nevertheless same-site co-infection appeared to have a stronger effect than compartmentalised co-infection in terms of decreasing the number of thoracic lymph node cells secreting IL4 in response to *L. sigmodontis* (Figs. 6:4A and 6:4B) and in response to the T cell mitogen Con A (Figs. Ap:2A and Ap:2B), as well as spontaneously *ex vivo* (Figs. Ap:1A and Ap:1B).

Although the anti-parasite immune responses were polarised correctly in compartmentalised co-infection, the shifts in the magnitude of these responses was associated with an enhanced ability of the host to defend itself against both *L. major*-induced pathology and *L. sigmodontis* infection. At day 40 PI more of the co-infected

animals were able to clear infection with *L. sigmodontis* (Fig. 6:6A) although this difference did not reach statistical significance. We measured the footpad lesions associated with *L. major* infection and found that a pre-existing *L. sigmodontis* infection protected mice against the total amount of pathology caused by *L. major* over the course of infection (Fig. 6:5). However it is unknown whether the changes in the number of parasite-specific cells secreting IL4 and IFNγ measured in these experiments at day 40 PI were a cause, or a consequence, of parasite numbers at this time point.

The average peak footpad lesion size in co-infected animals occurred 2 weeks later than in animals singly infected with *L. major* (Fig. 6:5). The average lesion size at day 14 post-infection was significantly decreased in co-infected animals (Fig. 6:5). We examined the immune responses against each pathogen, again in the draining lymph nodes for each pathogen compartment, at day 14 PI with *L. major*. However we did not detect any significant differences between the immune responses of co-infected and singly infected animals (Figs. Ap3 and Ap4). This was surprising because, in addition to alterations in *L. major*-induced pathology, we also observed a small decrease in the number of co-infected mice that contained adult filarial nematodes compared with singly infected animals at this time point. However there may have been changes in other components of the immune system, such as IL10 or TGFγ that may influence the course of infection (Chatelain *et al.*, 1999; Li *et al.*, 1999; Lawrence 2001) and that we did not measure in these experiments. Additionally the IL4 response mounted by singly infected mice in the thoracic lymph nodes, and the IFNγ response

in the popliteal lymph nodes, was weaker at this time point than at day 40 PI (Figs. Ap:3 and 6:2) and this may not have reached a necessary threshold for interactions between these two compartmentalised immune responses. Interestingly responses to Con A in the draining lymph nodes of each compartment at this time point indicate that there were increases in IFNγ-secreting cells in the thoracic lymph nodes (Fig. Ap:5A), and IL4-secreting cells in the popliteal lymph nodes (Fig. Ap:5B) of co-infected animals compared with animals singly infected with each corresponding pathogen. Although only the latter observation reached statistical significance (Mann Whitney-U test, $P<0.05$), this data provides support for the hypothesis that interactions were occurring between the two physically compartmentalised immune responses at day 14 PI.

There are many possible hypotheses why these physically compartmentalised immune responses were not independent. This could occur directly via the bloodstream from one compartment to the other. It is possible to detect serum cytokines in murine filarial infection (Rao *et al.*, 1996) and *L. major* infection (Huang *et al.*, 1998). Serum cytokines could reach the lymph nodes via the tissues and influence the differentiation of T cells via cytokine receptor signalling (O'Garra and Arai 2000). Alternatively the interactions observed may occur on a cellular basis. Cytokine secretion patterns are associated with differential chemokine receptor expression on T helper cells (Syrbe *et al.*, 1999) and the cross-regulatory nature of the two types of response generated by each pathogen may influence the levels of chemokine receptor expression that could

alter the number of antigen specific cells that home to the lymph nodes of each compartment after re-circulation.

Cellular interactions may also stem from the anti-parasite immune responses occurring in the spleen. The role of the spleen in primary infection is currently still unclear. The spleen appears to play a role in the clearance of some infections (Bonsack and Brown 1986; Favilo-Castillo *et al.*, 1999; Altamura *et al.*, 2001), but not all (Weiss 1978; Danko 1982; Magez *et al.*, 2002). Specifically in terms of *L. major* infection there is some evidence that the lymph nodes are crucial in controlling infection (Wilhelm *et al.*, 2002) although some role for the spleen cannot be ruled out by this study. Nevertheless, immune responses to infection located in different parts of the body can be found in the spleen and this may occur to protect the host from secondary subsequent infection with the same pathogen that may occur in a different part of the body from the site of primary infection. In the case of compartmentalised co-infection experiments parasite – specific splenic immune responses observed followed the same trends as those observed in each parasite compartment (Fig. 6:3). It is unclear from these studies where the interactions occurred that led to the observed shifts in the immune responses observed in co-infected animals. The role of the spleen in these observations could be examined by co-infecting splenectomised mice and measuring the immune responses to each pathogen in each compartment. It is unknown whether the cells involved in splenic immune response recirculate, however if so the follicles of the spleen may form a site in which the cells from the two compartments may meet and interact (Fig. 6:1).

The immunological interactions observed in these experiments may directly have altered the kind or amount of effector cell trafficking signals that resulted in accelerated adult nematode death and delayed pathology caused by *L. major*, perhaps by altering chemokine production by macrophages (Bonecchi *et al.*, 1998). Alternatively the observed effects of immunological interaction may occur because some cells types such as neutrophils appear to be involved in the killing of *L. sigmodontis* (Al-Qaoud *et al.*, 2001; Chapter 3) as well as in the inflammatory response mounted against *L. major* infection (Rodriguez-Sosa *et al.*, 2003). The delay in the development of the lesion caused by *L. major* may be due to the necessity to build up a level of adhesion molecules or a sufficient chemokine gradient to compete with that established by the filarial nematode infection. This hypothesis could be tested by examining the dynamics of cell recruitment to the footpad and thoracic cavity, over the time of co-infection alongside levels of adhesion molecules and chemokines. Nevertheless enhanced protection against both filarial infection and *L. major* induced pathology suggests that there may be an overall increase in effector cells that are common to both infections.

Interestingly the delay in lesion development caused by *L. major* has also been shown to occur in co-infection with established *Schistosoma mansoni* infection (Yoshida *et al.*, 1999; la Flamme *et al.*, 2002) although the delay in the study by Yoshida *et al.* (1999) did not reach statistical significance. Interestingly splenomegaly, a symptom of visceral *Leishmaniasis*, induced *by Leishmania infantum* in mice already co-infected with *Trichinella spiralis*, was reduced (Rousseau *et al.*, 1997). The data suggests that

the immune responses evoked by established helminth infection in general reduce the influx of cells to the site of *Leishmania* infection. It is unclear how these findings relate to *L. major* parasite numbers. However the reduction in footpad pathology may be due an immunological interaction that down-regulates cytokines that induce the expansion of effector cells, possibly because of early control of parasite numbers, or alternatively may be due to interaction of chemokine gradients preventing effector cells from entering the site of infection. None of the studies cited above examined the effect of *Leishmania* infection on the established helminth infection making it difficult to draw any conclusions about possible mechanisms of altered *L. major* pathology and accelerated adult filarial nematode death.

In conclusion this study has shown that immunological interactions can occur in co-infection, despite compartmentalisation of the different pathogens in the body. These interactions were important because they result in an acceleration of filarial adult nematode death, and appeared to protect the host against *L. major* induced footpad pathology. Further studies using this model are needed to understand more fully the immunological interactions that occur, the location in which they occur and also the mechanisms by which these interactions affect the parasites in this co-infection.

CHAPTER 7

The Majority of the Response Elicited Against *Wolbachia* Surface Protein in Filarial Nematode Infection is Due to the Infective Larval Stage

(Accepted for Publication in Journal of Infectious Disease – Lamb *et al.*, 2004)

7:1 Introduction

Filarial infection involves host exposure to both a nematode and its obligate intracellular bacterium, *Wolbachia*. *Wolbachia* are alpha-proteo bacteria that are commonly found to be endosymbiotic with a wide range of organisms (O'Neil 1997; Werren 1997). Their discovery in filarial nematode species in the 1970's was met with great interest (McLaren *et al.*, 1975). However it is only recently that studies into this symbiotic relationship have begun in earnest. *Wolbachia* have been found in most filarial species examined so far and reside in the hypodermis and reproductive tissues of filarial nematodes (Taylor and Hoerauf 1999).

As the potential significance of these bacterial parasites to the biology of filarial nematodes becomes more apparent (Taylor *et al.*, 2000), understanding the potential consequences of their interaction with the mammalian host becomes increasingly important. A key question is whether the immune response observed during filarial infection is directed against both organisms. We have therefore looked for evidence of immune responses to the intracellular bacteria of filarial parasites by investigating the pattern of antibody responses against the *Wolbachia* Surface Protein (WSP).

We started the analysis by evaluating antibody responses from individuals in an area endemic for *Brugia malayi*. Findings from the human study were further investigated by experimental infections with the rodent filaria *Litomosoides sigmodontis*. Interestingly our data from both human and murine studies show that antibody responses against WSP are made during natural infection and suggest that the majority of this response is induced by the infective larval stage. This provocative finding is supported by data showing that, per gram of nematode, the WSP in the L3 stage of *L. sigmodontis* induces the strongest immune response of all the developmental stages.

7:2 RESULTS

7:2:1 Recombinant L. sigmodontis Wolbachia *Surface Protein recognizes WSP in adult* L. sigmodontis *extract.*

We generated recombinant WSP from the *Wolbachia* of *L. sigmodontis* (*Ls*WSP) and from the *Wolbachia* of *B. malayi* (*Bm*WSP) to allow us to investigate the pattern of anti-*Wolbachia* immune responses in human and murine infection. To verify the identity of the recombinant protein we generated antibodies to *Ls*WSP by vaccinating BALB/c mice with *Ls*WSP in Freunds' adjuvant. Western blot analysis confirmed that the recombinant *Ls*WSP was similar to native *Ls*WSP because the antibodies generated against *Ls*WSP recognized a protein of around 24 kDa in detergent soluble adult *L. sigmodontis* extract (Fig. 7:1). This band was not present in PBS soluble adult *L. sigmodontis* extract consistent with the fact that the recombinant protein was

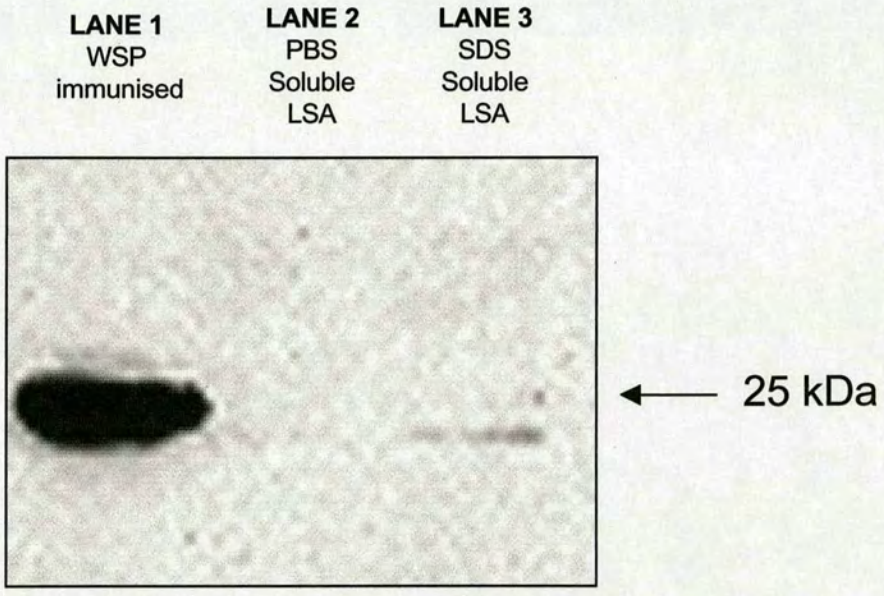


Figure 7:1 Verification of recombinant *Ls*WSP. The western blot was probed with sera from BALB/c mice immunised with *Ls*WSP in Freund s adjuvant. The antibodies recognised the recombinant LsWSP the mice were immunised with (24.5 kDa, Lane 1) and a band in detergent soluble adult *L. sigmodontis* extract (24.5 kDa, Lane 3) but not PBS soluble adult *L. sigmodontis* extract (Lane 2).

insoluble. This is in contrast to a previous study which indicated that WSP from *Diofilaria immitis* (*Di*WSP) could be detected by ELISA in PBS soluble nematode homogenates (Marcos-Atxutegi *et al.*, 2003).

7:2:2 Human Responses to Bm*WSP in* B. malayi *infection.*

We investigated whether individuals living in an area endemic for Brugian lymphatic filariasis showed evidence of immune responses to *Wolbachia* proteins. We looked at total IgG antibody responses to recombinant *Bm*WSP in 3 generally accepted different clinical groups (Yazdanbakhsh *et al.*, 1993). One hundred and four human sera samples from residents of two different areas of Sumatra, Indonesia were selected for testing. These samples were divided into three groups based on parasitological and clinical status as previously described (Kurniawan *et al.*, 1993; Yazdanbakhsh *et al.*, 1993; Sartono *et al.*, 1995). European control samples were taken from volunteers at Edinburgh University, Scotland. ANOVA was performed to detect differences among the antibody responses of three human clinical groups against *Bm*WSP, *B. malayi* L3 and adult extracts, and also *B. pahangi* ladder protein. This was followed by Tukey's multiple comparison tests to analyze pairwise comparisons. Optical density values multiplied by 100 were logarithmically transformed prior to these analyses to normalize the data and allow parametric analyses to be performed. This transformation was not sufficient to normalize the values against *B. pahangi* ladder protein and thus equivalent non-parametric tests were used to analyze the transformed values against this protein (the Kruskal-Wallis test followed by Dunn's multiple-comparison test).

The antibody response to *Bm*WSP was different amongst these groups (ANOVA, $P<0.05$) (Figure 7:2A). The chronic pathology group demonstrated significantly greater responses to *Bm*WSP than the endemic normal group (Tukey's pairwise multiple comparison test, $P<0.05$). This trend was similar to the antibody response to the larval stage of *B. malayi* (ANOVA, $P=0.01$) (Fig. 7:2B) but not the adult stage, which did not differ statistically among the clinical groups (ANOVA, $P=0.347$) (Fig. 7:2C). As a control we tested responses to another recombinant protein (*B. pahangi* ladder protein) that is also nematode derived (Paxton *et al.*, 1993) (Fig. 7:2D). The antibody responses to the ladder protein exhibited a similar distribution to the anti-*Bm*WSP and anti-L3 responses such that they differ statistically between the different clinical groups tested (Kruskal-Wallis, $P<0.001$). Both the chronic pathology and the asymptomatic microfilaraemic groups had statistically significant higher responses than the endemic normal group (Dunn's multiple comparison test, both $P<0.05$) (Fig. 7:2D).

General Linear Modeling (GLM) was used to analyze the logarithmically transformed optical density readings indicating the human antibody responses (Grafen and Hails, 2002). GLM uses regression to partition the variation in an observed response between different possible variables (in this case responses to L3 or adult *B. malayi*, age, gender and location of residence of the sampled individuals). Analysis of the residuals from the GLMs confirmed that the transformed data accorded with the normality and homogeneity of variance assumptions of parametric tests. In each model, variables that did not significantly correlate with anti-*Bm*WSP responses were removed before re-

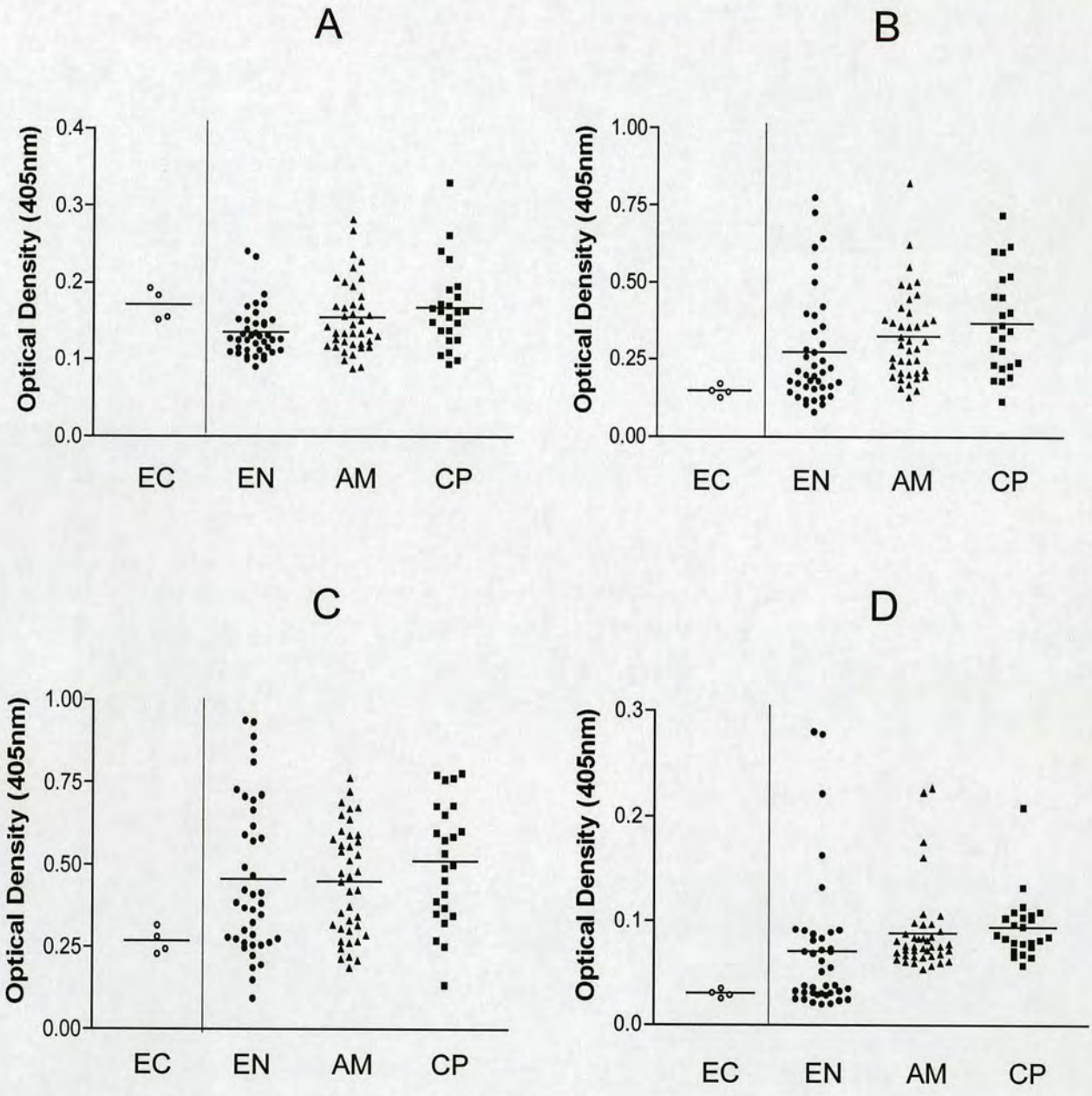


Figure 7:2 Total IgG responses to *Bm*WSP (A), *B. malayi* L3 extract (B), *B. malayi* adult extract (C) and *B. malayi* ladder protein (D) in human infection with *B. malayi*. Subjects were grouped into "endemic normal" (EN) with undetectable infection or pathology, asymptomatic microfilaraemics (AM) with circulating microfilariae and no signs of pathology and those with clinical pathology (CP). European control sera were included for comparison (EC). Each data point refers to one person. Bars represent the mean of each group. A 1:400 dilution of sera is shown. All optical density values were multiplied by 100 and then logarithmically transformed before performing statistical analyses.

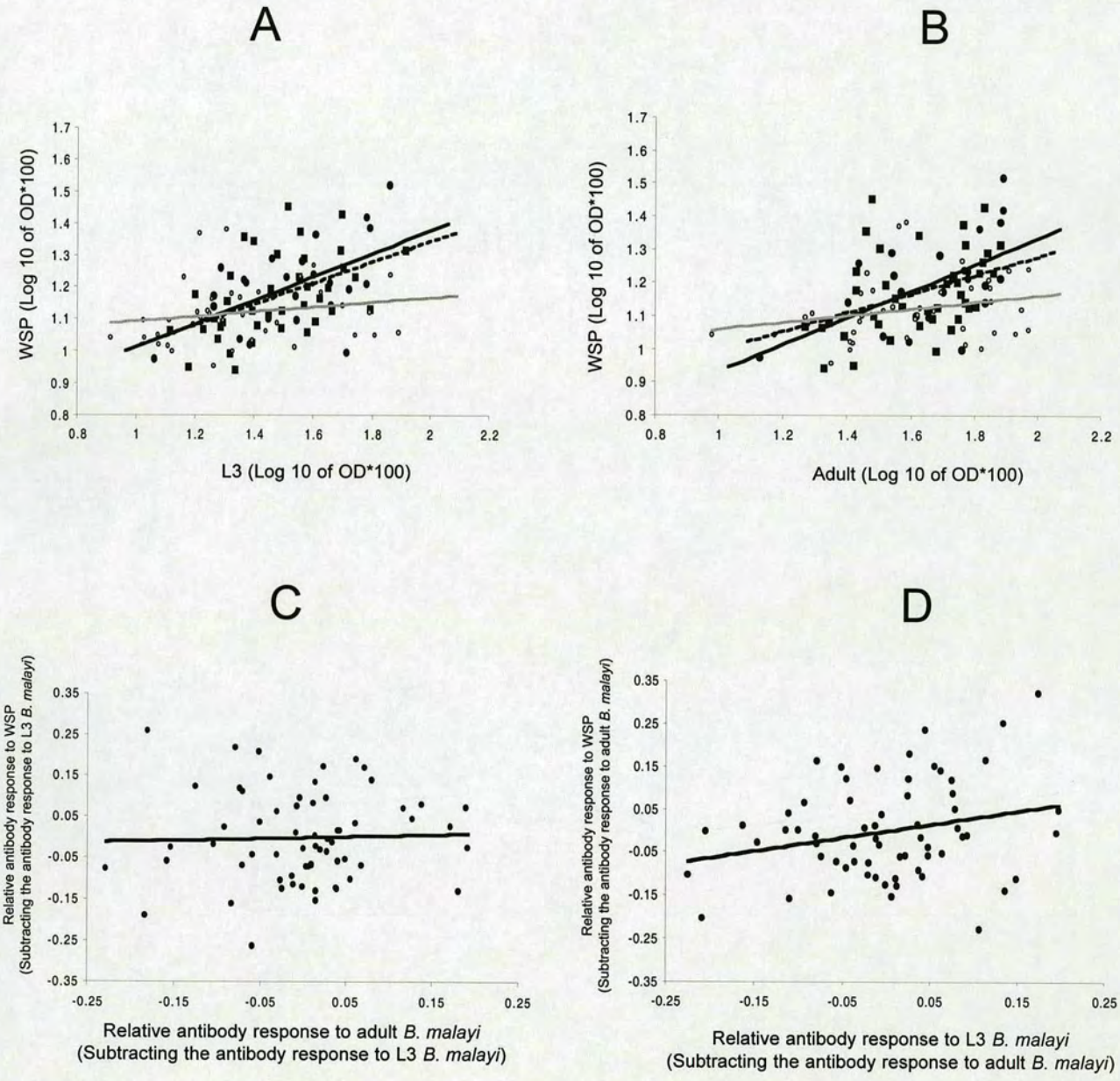
running the model. Using this method, GLM can disentangle the non-contributing variables from the anti-L3 and anti-adult responses that serve as predictors of responses to *Bm*WSP. Significant P-values are from the minimal model (only including significant variables); non-significant P-values are from the models from which they were removed.

General Linear Modelling (GLM) was used to examine whether the within group variation in the antibody response to *Bm*WSP could best be explained by responses to *B. malayi* larvae, *B. malayi* adults or the age, sex, clinical status or location of the people tested. Generally, anti-*Bm*WSP responses were positively correlated with responses to both L3 and adult stages of *B. malayi* (Figs. 7:3A and 7:3B: anti-L3 response $F_{1,103}=22.88$, $P<0.001$; anti-adult response $F_{1,103}=17.75$, $P<0.001$). The patterns in Figs. 7:3A and 7:3B could be due to *Bm*WSP responsiveness arising from exposure to L3 stages alone, to adult stages alone, or to both stages of *B. malayi*. To test which of these parasite stages were responsible for *Bm*WSP responsiveness we asked whether anti-L3 responses and anti-adult responses are independently associated with anti-*Bm*WSP responses.

The method employed to visualize the GLM result indicating that antibody responses to *B. malayi* L3, rather than adults, are correlated with anti-*Bm*WSP responses is as follows. We asked whether strong responses to *Bm*WSP and the L3 stage are still correlated once the contribution of anti-adult responses has been subtracted. We removed the endemic normal group from this analysis because in this group there was

Figure 7:3 The total IgG response to *Bm*WSP plotted against the response to L3 extract (A) and *B. malayi* adult extract (B). White circles represent endemic normals. Squares represent asymptomatic microfilaraemics. Black circles represent subjects presenting with chronic pathology. Solid lines represent the best-fit line through the data points for the elephantiasis group. Likewise the dashed and gray lines represent the asymptomatic microfilaraemic and endemic normal groups respectively. The optical density data were multiplied by 100 and then logarithmically transformed so that the values accorded with the normality and homogeneity of variance assumptions of parametric tests.

Plotted values in Figs. 2C and 2D are of the levels of disproportionate responses mounted by individuals to adult *B. malayi* or L3 *B. malayi* respectively (x-axes) each plotted against the respective disproportionate response to *Bm*WSP (y-axes). The positive and negative values represent whether there was a positive or negative response (residuals) compared to the best fit lines through the following plots: Fig. 2C, x-axis; anti-adult responses against anti-L3 responses; Fig. 2C, y-axis; anti-*Bm*WSP responses against anti-L3 responses; Fig. 2D, x-axis; anti-L3 responses against anti-adult responses; Fig. 2D, y-axis; anti-*Bm*WSP responses against anti-adult responses. The lines represented on Figs 2C and 2D are of the best least-squares fits through the plotted points.


A
WSP (Log 10 of OD*100)
L3 (Log 10 of OD*100)
B
WSP (Log 10 of OD*100)
Adult (Log 10 of OD*100)
C
Relative antibody response to WSP
(Subtracting the antibody response to L3 B. malayi)
Relative antibody response to adult B. malayi
(Subtracting the antibody response to L3 B. malayi)
D
Relative antibody response to WSP
(Subtracting the antibody response to adult B. malayi)
Relative antibody response to L3 B. malayi
(Subtracting the antibody response to adult B. malayi)

no correlation between the anti-*Bm*WSP responses and either the anti-L3 or anti-adult *B. malayi* responses. In the remaining data when these responses are plotted against *B. malayi* L3, few individuals responded to *Bm*WSP exactly as predicted by the best-fit line (Figs. 7:3A). We plotted the distance these responses fall away from the best-fit line (positive and negative residuals for strong or weak responses to *Bm*WSP for a given response to L3) against the positive and negative residuals from a plot of anti-*B. malayi* L3 against anti-adult responses. The latter residuals represent strong or weak responses to *B. malayi* L3 for a given level of anti-adult response- the equivalent of subtracting anti-adult responses from anti-L3 responses. For all plots the logarithmically transformed optical density data was used. This method was repeated to determine whether responses to *Bm*WSP correlate with responses to adult stages once the anti-L3 responses had been removed.

There was no evidence that the responses to adult *B. malayi* and *Bm*WSP were associated when the responses to L3 were controlled for (General Linear Model $F_{1,63}$=0.08, P=0.779) (Fig. 7:3C). In contrast, the responses to L3 were still associated with responses to *Bm*WSP when the responses to adult *B. malayi* were controlled for (General Linear Modelling $F_{1,63}$=4.29, P=0.043) (Fig. 7:3D). Thus the data suggest that anti-*Bm*WSP responses arise through exposure to L3 stages alone and that exposure to adult *B. malayi* does not contribute to anti-*Bm*WSP responses.

We were able to visualize the result of the GLM by showing that strong responses to L3, over and above the anti-adult responses, were correlated with anti-*Bm*WSP

responses (Fig 7:3D). This was not the case for anti-adult responses (Fig. 7:3C). The association between anti-*BmWSP* and anti-adult *B. malayi* responses (Fig. 7:3B) arises because responses to adult and L3 stages are correlated (General Linear Model $F_{1,103}=311.45$, $P<0.001$), possibly due to cross-reactivity between the two stages.

The correlation between the magnitude of the anti-filarial response and the magnitude of the anti-*BmWSP* response was identical in both the chronic pathology and the asymptomatic microfilaraemic groups when tested for differences in slope and intercept (all $P<0.5$), even upon controlling for the potentially confounding effects of age, gender and location of people sampled ($P>0.05$ in all cases). However among the endemic normal group *Bm*WSP responsiveness was not significantly related to responsiveness to either *B. malayi* stage (General Linear Modelling, anti-L3 response $F_{1,39}=0.25$, $P=0.62$; anti-adult response $F_{1,39}=0.04$, $P=0.847$). The variation was best explained by the location of endemic normal group (General Linear Model $F_{1,39}=5.26$, $P<0.05$).

7:2:3 Mouse Responses to Ls*WSP in* L. sigmodontis *infection.*

GLM analysis of the human responses to *Bm*WSP led to unexpected and provocative results concerning the role of the L3 stage in the immune responses against *Wolbachia*. To ask more specific questions regarding the induction and maintenance of anti-*Wolbachia* responses we used a murine model of filarial infection that permits the full developmental cycle of the parasites (for review see Marechal *et al.*, 1996). We analyzed the total IgG response to *Ls*WSP in BALB/c mice challenged with *L.*

sigmodontis. We found that in a primary infection, the majority of mice mounted a low but statistically significant response to *Ls*WSP (Mann Whitney-U Test $P<0.05$) at 20 days post-infection in comparison with naive animals (Fig. 7:4A). This significant difference was observed in two separate experiments. This response fell to background levels at day 40 but rose again at day 60 as the adults reached maturity and began reproducing. In contrast, there were significant responses to *L. sigmodontis* adult antigen (LSA) at all time points from day 20 onwards (Mann Whitney-U Test, $P\leq0.01$) which increased as the infection progressed and peaked at 60 days post-infection, when adult parasites had reached patency and began to die (Fig. 7:4B).

The responses to *Ls*WSP induced by adults in *L. sigmodontis* primary infection were highly variable and lower than might be expected if *Wolbachia* antigen is released predominantly upon parasite death. One cause of the variability may be due to the onset of the production of Mf as in primary infections with *L. sigmodontis* only 50% of BALB/c mice become microfilaraemic (Marechal *et al.*, 1996). Alternatively, variation in the number of nematodes surviving to adulthood may contribute to the variation in these experiments. We therefore decided to examine more closely the responses to WSP using a more homogenous system in which adult parasites are directly implanted into the peritoneal cavity of BALB/c mice (MacDonald *et al.*, 1998). Implantation of Mf-producing adult parasites induced a very weak, although statistically significant response against *Ls*WSP (Mann Whitney-U Test, $P<0.01$; Student's t-test, $P<0.01$) compared to naive animals, whereas large amounts of anti-*L. sigmodontis* adult IgG antibodies were produced (Mann Whitney-U Test, $P<0.01$;

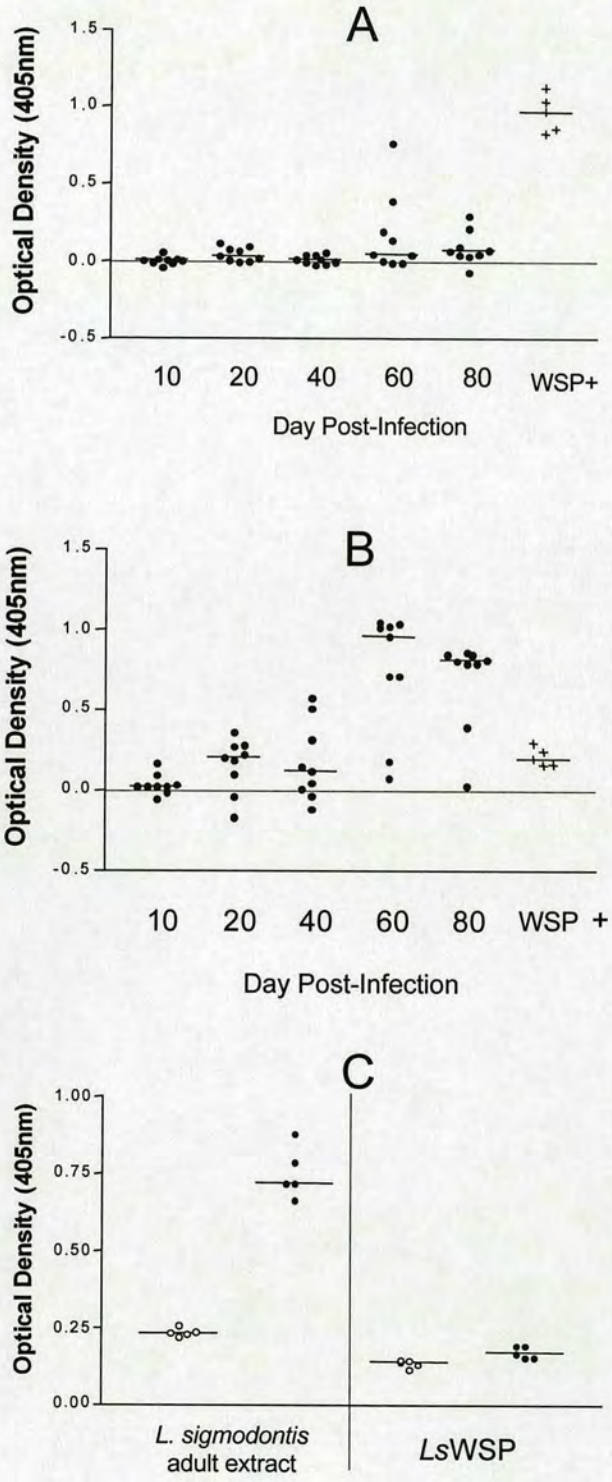


Figure 7:4 The total IgG response to *Ls*WSP (A) and adult *L. sigmodontis* extract (B) made by BALB/c mice challenged with a primary infection of *L. sigmodontis*. At all points after infection the average response of all the naive animals was subtracted from the response of each individual infected animal at the relevant time point. Two experiments containing 5 naive animals and 5 infected animals are represented at each time point examined. Each data point represents one animal. A 1: 200 dilution of sera is shown. The bars on each graph show the median of each group. Antisera to recombinant *Ls*WSP (WSP+) were included in all ELISAs as a positive control for WSP. Mice implanted with adult *L. sigmodontis* for 21 days were tested for antibody responses to adult *L. sigmodontis* extract and *Ls*WSP (C). Open circles represent naive mice that underwent surgery but were not implanted.

Student's t-test, P<0.0001) (Fig. 7:4C). This data indicates that Mf-producing adults alone may not be a major inducer of anti-WSP responses.

7:2:4 Mouse Responses to Ls*WSP in different parasite life cycle stages.*

Wolbachia are vertically transmitted and thus this bacterium is present in all stages of filarial nematodes (for review see Taylor and Hoerauf 1999). Our studies thus far indicated that the *Wolbachia* inside the infective larval stage are immunogenic (Figs. 7:3A and 7:4A) and potentially the most important stage in inducing anti-WSP responses. In an attempt to further clarify the contribution of the *Wolbachia* within the adults and microfilariae to the observed response to *Ls*WSP, we injected extracts of each of these stages of *L. sigmodontis* in emulsions of Freud's adjuvant and looked for responses to *Ls*WSP.

Strikingly, the only mice that showed responses to *Ls*WSP significantly above those of the control animals were those injected with L3 extract (Mann Whitney-U Test, P<0.05) (Fig. 7:5A). As controls for this experiment, the sera from the mice were tested for antibody responses to the L3 stage, adults and Mf. As expected, all mice injected with L3 extract produced antibodies to this antigen as determined by ELISA (Mann Whitney-U Test, P<0.05) (Fig. 7:5B). All mice injected with Mf extract, except for one, produced cross-reactive antibodies to L3 extract (Mann-Whitney-U Test, P< 0.05) (Fig. 7:5B). The mice injected with adult extract produced antibodies to adult extract as well as L3 extract (both Mann Whitney-U Test, P<0.05) (Fig. 7:5C), confirming the result of our human studies that the L3 and adult stage of filarial

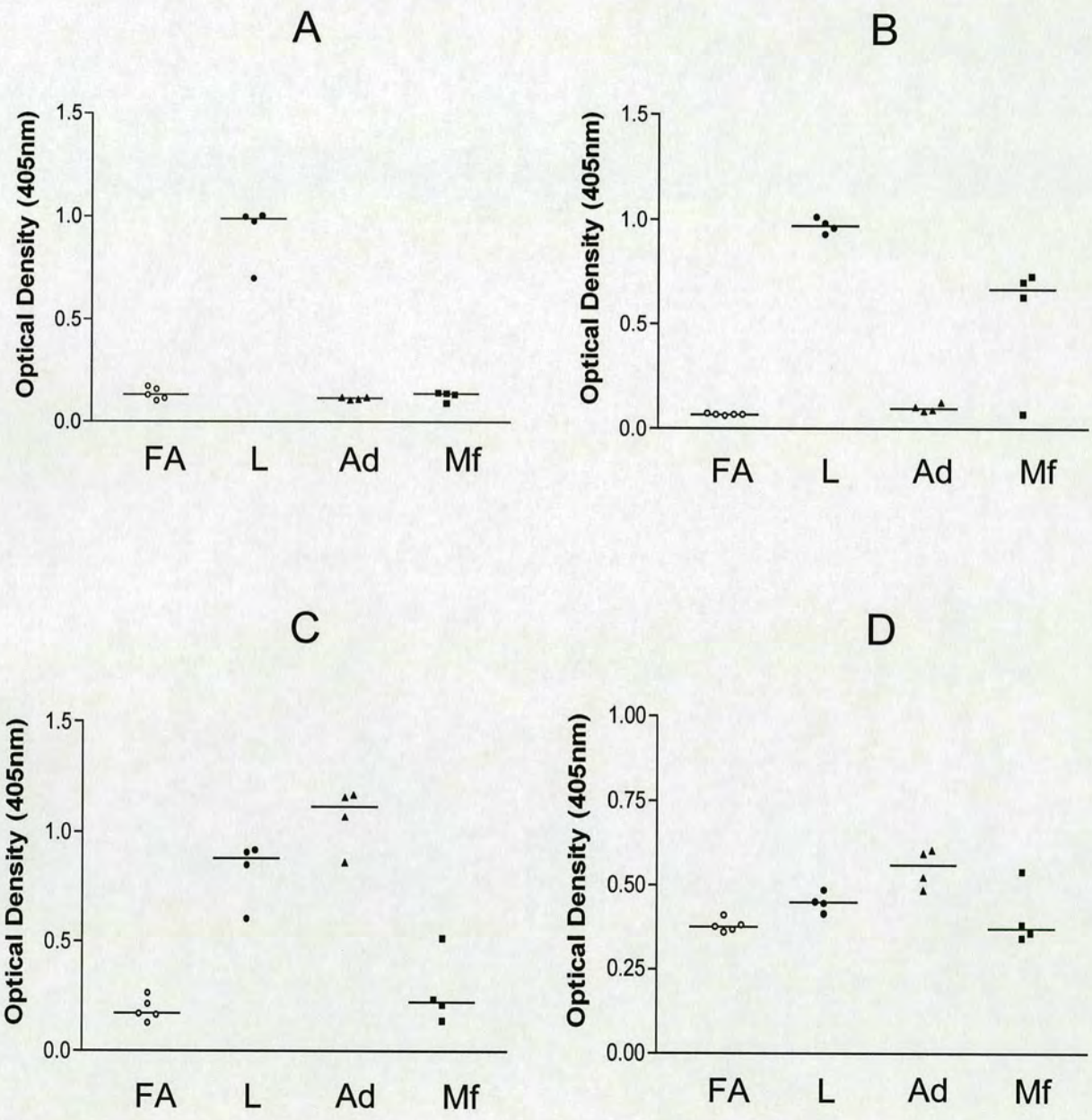


Figure 7:5 Antibody responses against *Ls*WSP (A), L3 extract (B), adult extract (C) and Mf extract (D) elicited by BALB/c mice injected subcutaneously with extracts of different stages of *L.sigmodontis*. Only data from sera taken 4 weeks after the final dose of extract are shown for clarity. A 1:200 dilution of sera is shown. Each data point refers to an individual animal. The bars represent the median of each group. FA, injected with PBS and FA emulsion, L, Injected with L3 extract and FA emulsion, Ad, injected with adult extract and FA emulsion, Mf, injected with microfilarial extract and FA emulsion.

nematodes are highly cross-reactive. Mice injected with Mf extract recognized the L3 antigen (Fig. 7:5B) better than the Mf antigen (Mann Whitney-U Test, $P>0.05$) (Fig. 7:5D). This result, although difficult to explain, was observed in two separate experiments. Interestingly, the single mouse injected with Mf extract that produced a recall response to Mf extract was the mouse that did not produce a cross-reactive antibody response to L3 extract. The mice injected with both larvae and adults produced cross-reactive antibodies to Mf extract (Fig. 7:5D) (Mann Whitney-U Test, both $P<0.05$) showing that the lack of antibodies to Mf extract in mice injected with this antigen was not due to the failure of the ELISA assay. These results are not surprising since Mf are juvenile larvae that are likely to share antigenic components with the L3 stage and the adult extract was made from mixed adults, containing gravid Mf-producing females.

7:3 Discussion

The data from this study demonstrate that *Wolbachia* are an immunogenic component of filarial nematodes and that responses are made against *Wolbachia* in both human and murine filarial infection. Anti -WSP responses have recently been shown to be produced in *Dirofilaria immitis* infection of cats (Bazzocchi *et al.*, 2000a) and *B. malayi* infection of Rhesus monkeys (Punkosdy *et al.*, 2001) indicating that responsiveness to WSP is a feature of filarial nematode infection.

Previously it has been hypothesized that the death of adult filarial nematodes is largely responsible for the release of *Wolbachia* that subsequently causes the damaging inflammatory immune responses observed in elephantiasis patients (Taylor 2002). Indeed death of filarial nematodes significantly increases the levels of *Wolbachia* DNA in the bloodstream of humans (Keiser *et al.*, 2002). Supporting this, our data indicate that human immune responses to *Bm*WSP, the major surface protein of *Wolbachia* within *B. malayi,* are correlated with anti-filarial adult responses (Fig. 7:3B). Additionally, the death of adult parasites in *L. sigmodontis* infection appears to induce an immune response to *Wolbachia* (Figs. 7:4A). However anti-*Bm*WSP responses in humans were also correlated with anti-L3 stages (Fig. 7:3A) and GLM analyses indicated that responses to *Bm*WSP were more likely to be generated by the L3 stage than the adult stage of *B. malayi*. Indeed, further experiments with the *L. sigmodontis* murine model, strongly supported this finding as, per gram of parasite, WSP within the L3 stages induced the strongest response (Fig. 7:5A). Thus the positive correlation between anti-*Bm*WSP and anti-adult responses are likely to be due to cross-reactivity between L3 and adult stages of the parasite as shown in Fig. 7:5C.

We statistically tested other factors, such as the age, sex or the location of the people tested, that could be responsible for our finding that anti-*Bm*WSP responses are mainly generated from the L3 stage. Anti-L3 responses increase with age (Day *et al.*, 1991) and it is also well documented that people with elephantiasis tend to be older (Michael *et al.*, 1996; Witt and Ottesen 1991). Therefore it was possible that variation in age rather than responses to larvae *per se* could better explain the observed responses to

*Bm*WSP. However responses to the L3 stage of *B. malayi* were more tightly correlated with responses to *Bm*WSP than any of these variables.

The endemic normal group was the only clinical group tested in which responses to *Bm*WSP did not positively correlate with responses to the L3 stage of *B. malayi*. This group had the same amount of within-group variation in antibody responses to the L3 stage, but less variation in anti- *Bm*WSP responses compared with the other two clinical groups (Bartlett's test statistic for homogeneity of variance on optical density values = 8.53 $P<0.05$). Since the variation in responsiveness to L3 antigen, and thus probably exposure to infective mosquito bites is comparable in all three clinical groups tested, low biting rate is an unlikely explanation for why there is little variation in responses to *Bm*WSP among endemic normals. However the immune systems of the majority of this group will not have been exposed to dead adult filarial nematodes. It is possible that the death of adult *B. malayi* may be required to permit strong antibody responses to the WSP in incoming L3 stages.

Primary infection of *L. sigmodontis* in BALB/c mice indicates that a response to WSP can be induced prior to adult exposure (Fig. 7:4A), but this response (observed at day 20 post-infection), although statistically significant compared to naive animals, was weak. This may be because only a small number of larvae (25 L3 stages) were used to set up primary *L. sigmodontis* infection. When adult stages reached patency and started to die (day 60 post-infection) some animals produced stronger responses to *Ls*WSP. The data from both the implantation studies with *L. sigmodontis* as well as the

injection of parasite extracts in adjuvant suggest that WSP is not a major immunogen when an individual is exposed to adult or microfilarial stages in the absence of exposure to L3s. The extract immunization studies would favor the hypothesis that the L3 stage of the parasite generates the strongest anti-WSP responses because it contains the highest amount of *Wolbachia* per gram of nematode.

Collectively the data from both human and mouse studies suggest that exposure to both L3s and adults are required to generate a strong anti-WSP response. The requirement for the L3 stage is supported by the GLM of human antibody responses to WSP and is made considerably more convincing by the murine studies which demonstrate that infective larvae are intrinsically the most immunogenic when it comes to anti-WSP responses. The requirement for the adult stage is also suggested by the significantly lower responsiveness in endemic normals who may not harbor adult parasites and are certainly less likely to be exposed to large numbers of dying parasites. This is further supported by primary infection of mice with *L. sigmodontis* in which robust responses to WSP were not observed until mature adults were present. We would hypothesize that the death of adult parasites are required to generate the immunological environment which allow anti-WSP responses to develop. However, once these responses are initiated, it is the incoming larvae that are the major inducers of the response. The significantly stronger responses to *Bm*WSP in those with chronic pathology may arise due to the immune hyperactivity that is commonly observed in this clinical group (Maizels and Lawrence 1991; Maizels *et al.*, 1991).

This study using a combination of human and animal studies provides evidence that the infective larval stage is a key player in the generation and maintenance of an anti-*Wolbachia* response. It has been proposed that *Wolbachia* induced immune responses may cause filarial pathology particularly at the time of adult parasite death (Taylor 2002; Keiser *et al.*, 2002; Saint-Andre *et al.*, 2002). Our data support a role for adult death in anti-*Wolbachia* responses but surprisingly suggest that it is the L3 stage that may be more critical in driving our maintaining that response. As increasing efforts towards designing vaccines against both Elephantiasis and River Blindness are underway, this study emphasizes the importance of focusing on transmission-blocking strategies that reduce exposure to the L3 stages, not only to prevent transmission but perhaps as key step in reducing pathology.

CHAPTER 8

Do anti-*Wolbachia* immune responses have any effect on the biology of filarial nematodes?

8:1 Introduction

Wolbachia are intracellular bacteria found in the tissues of most species of filarial nematodes (Taylor and Hoerauf 1999). They have a similar phylogeny to filarial nematodes (Bandi *et al.*, 1998) and appear to be ubiquitous within individual filarial nematode species studied (Taylor and Hoerauf 1999) indicating that they may have co-evolved with their nematode hosts.

The requirement for *Wolbachia* in filarial nematode survival is still not clear. Elimination of *Wolbachia* from nematode tissues using the antibiotic tetracycline has been shown to reduce the fertility of some species of filarial nematodes (Bandi *et al.*, 1999; Hoerauf *et al.*, 1999) as well as the development of the L3 stage both *in vitro* (Smith and Rajan 2000) and *in vivo* (Bosshardt *et al.*, 1993). Tetracycline-mediated removal of *Wolbachia* has also been shown to be macrofilaricidal against the bovine filarial nematode *Onchocerca ochengi* (Langworthy *et al.*, 2000). However the possibility that these effects are due to the release of toxic products from dead and dying *Wolbachia*, rather than the absence of live *Wolbachia per se*, has not yet been eliminated.

The survival of filarial *Wolbachia* is dependent on the survival, successful mating and transmission of filarial nematodes in the mammalian host. By promoting some, or all of these aspects in their filarial host, *Wolbachia* would maximise its own chances of transmission. Immune responses to *Wolbachia* can occur in human filarial infection (Simon *et al.*, 2003; Lamb *et al.*, 2004), feline filarial infection (Bazzocchi *et al.*, 2000a) and *B. malayi* infection in monkeys (Punkosdy *et al.*, 2001). The anti-*Wolbachia* immune responses observed in filarial infection could simply be a by-product of dying filarial nematodes, in particular incoming L3 stages (Lamb *et al.*, 2004). However the manipulation of the host immune system by filarial nematodes is a key determinant of their survival within the mammalian host (Maizels and Lawrence 1991; Maizels *et al.*, 1991). Therefore it is also possible that anti-*Wolbachia* immune responses may positively influence the survival of filarial nematodes and are evoked by *Wolbachia* in order to maximise their own survival. Further *Wolbachia* are more abundant in female filarial nematodes than in male filarial nematodes (Taylor and Hoerauf 1999). Therefore any observed effect of anti-*Wolbachia* immune responses may differentially effect male and female nematode establishment.

We have generated anti- *Wolbachia* surface protein (WSP) immune responses in BALB/c mice and used these animals to examine whether anti-WSP immune responses have any effect on the establishment of the rodent filarial nematode *Litomosoides sigmodontis* (Diagne *et al.*, 1990). It is currently unknown whether anti-*Wolbachia* immune responses in filariasis occur under type 1 conditions that are normally evoked by bacteria, or type 2 conditions that normally prevail in filarial

infection (Ottesen 1992; Lawrence and Devaney 2001). Therefore we have undertaken these experiments generating anti-WSP immune responses under type 2 conditions alone using the adjuvant alum, and under mixed type 1 and type 2 conditions using Freund's adjuvant.

We have found that anti-*Ls*WSP immune responses generated under mixed type 1 and type 2 conditions, but not type 2 conditions alone, promoted the establishment of *L. sigmodontis*. Interestingly when low numbers of nematodes were recovered in the presence of anti-*Ls*WSP responses the female biased sex ratio observed in control animals did not occur. This pattern was observed regardless of the adjuvant used to generate the anti-WSP immune responses.

8:2 Results

*8:2:1 Generation of anti-*Ls*WSP immune responses*

We vaccinated animals 3 times with *Ls*WSP at one-month intervals. Control animals were vaccinated with Freund's adjuvant alone, or with hen egg lysozyme (HEL) precipitated with alum, using the same protocol. HEL was used as a control protein as it is not thought to cross-react with filarial proteins. Two experiments were carried out using alum adjuvant and two experiments were carried out using Freund's adjuvant. To assess the efficacy of this vaccination protocol we carried out ELISA's on the sera of these animals prior to infection and used antibody production against *Ls*WSP as a read out for anti-*Ls*WSP immune responses. Most animals produced anti-WSP

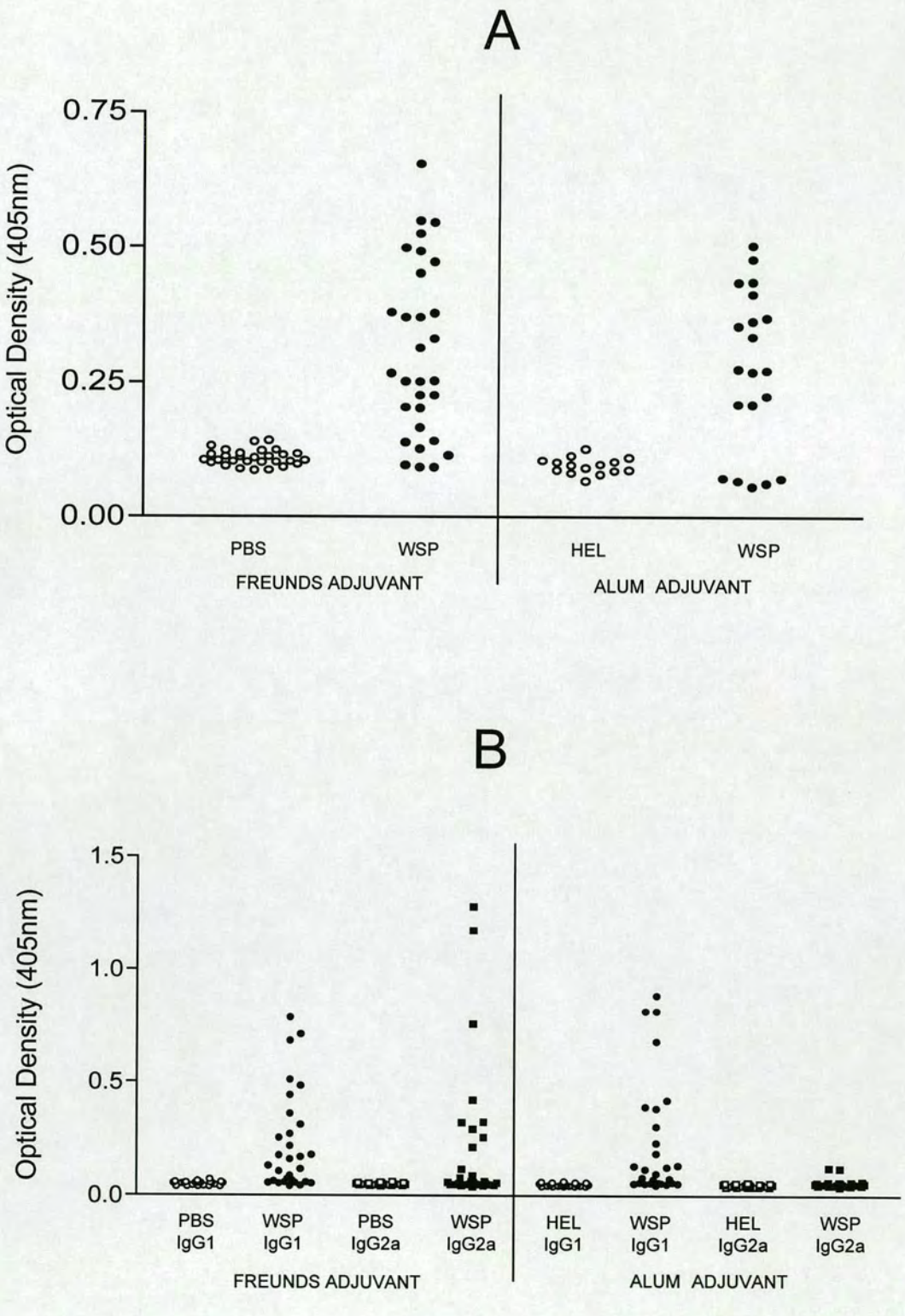


Figure 8:1. Animals injected with *Ls*WSP produced antibody responses to *Ls*WSP. Total IgG antibody responses are shown in A. IgG1 and IgG2a responses are shown in B. Sera was diluted 1:200 to measure total IgG and IgG1 responses and diluted 1:100 to measure IgG2a responses.

antibody responses although there were some non-responders in each adjuvant group (Fig. 8:1A). To assess the type of anti-*Ls*WSP immune responses generated we measured IgG1 and IgG2a levels within these anti-*Ls*WSP responses as markers of type 2 and type 1 responses, respectively. As expected *Ls*WSP injected in alum gave predominantly a type 2 response as indicated by the lack of anti- *Ls*WSP IgG2a, whereas Freund's adjuvant induced antibodies indicative of both type 1 (IgG2a) and type 2 (IgG1) responses (Fig. 8:1B).

8:2:2 Anti-WSP immune responses generated under mixed type 1 and type 2 conditions promote the establishment of L. sigmodontis.

We assessed the recovery of adult *L. sigmodontis* 60 days post-infection in all four experiments carried out. Square root transformation of the number of parasites recovered from each group of mice was undertaken prior to met-analysis of the data. There were no interactions observed between experiment and treatment for either adjuvant (ANOVA $P>0.05$ in both cases). More adult parasites were recovered from animals vaccinated with *Ls*WSP in Freund's adjuvant compared to animals vaccinated with Freund's adjuvant alone (ANOVA $P=0.028$) (Fig. 8:2A). Vaccination with *Ls*WSP precipitated in alum did not have any effect on the number of adult nematodes recovered compared with animals vaccinated with the control protein HEL (ANOVA $P>0.05$) (Fig. 8:2B).

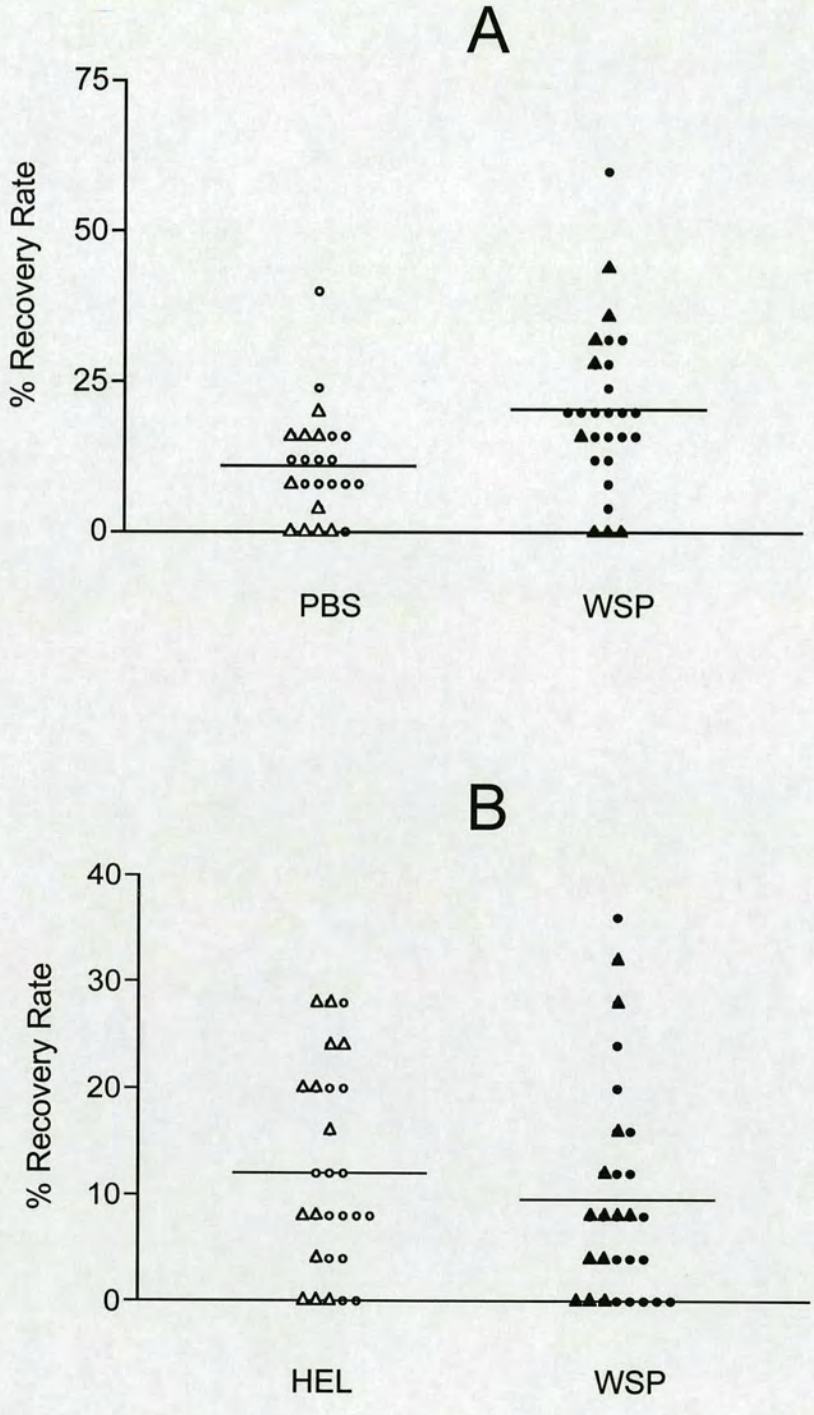


Figure 8:2. The recovery of filarial nematodes is greater in animals with pre-existing anti-WSP responses generated with Freud's adjuvant, but not alum. The adult nematode recovery rate is of animals injected with Freund's adjuvant is shown in A. Animals injected with Alum are shown in B. In both cases the circles represent data points from one experiment and the triangles represent data points from another experiment. The bars represent the mean recovery rate for each group of animals.

*8:2:3 There is no bias in the sex ratio of nematodes recovered from animals with pre-existing anti-*Ls*WSP immune responses.*

The tissues of male filarial nematodes contain less *Wolbachia* than those of female filarial nematodes. To assess whether anti-*Ls*WSP responses have differential effects on the establishment of male and female nematodes we examined the sex of the adult nematodes recovered from animals in both *Ls*WSP and sham vaccinated groups. The increase in nematode establishment in animals vaccinated with *Ls*WSP in Freund's adjuvant appeared to be due to an increase in both male and female nematodes compared with sham-vaccinated animals (Fig. 8:3A). However only the increase in the number of female nematodes reached statistical significance (ANOVA P=0.026). The number of males recovered from *Ls*WSP vaccinated and sham vaccinated animals was not statistically different (ANOVA P=0.06) (Fig 8:3A). As expected from the overall nematode establishment, there were no differences in either the number of males, or the number of females, recovered from animals vaccinated with *Ls*WSP in alum compared with animals vaccinated with HEL in alum (ANOVA P>0.05 in both cases) (Fig. 8:3B).

We examined the sex ratio of nematodes that were able to establish in *Ls*WSP vaccinated animals. We plotted the observed male skew against the total number of adult filariae recovered from each animal (Fig. 8:4). This data indicates that in control animals, when conditions are not favourable and only a few parasites are able to establish in an animal, most of these parasites are females (Figs. 8:4A and 8:4B). However in animals vaccinated with *Ls*WSP in either Freund's adjuvant (Fig. 8:4A) or

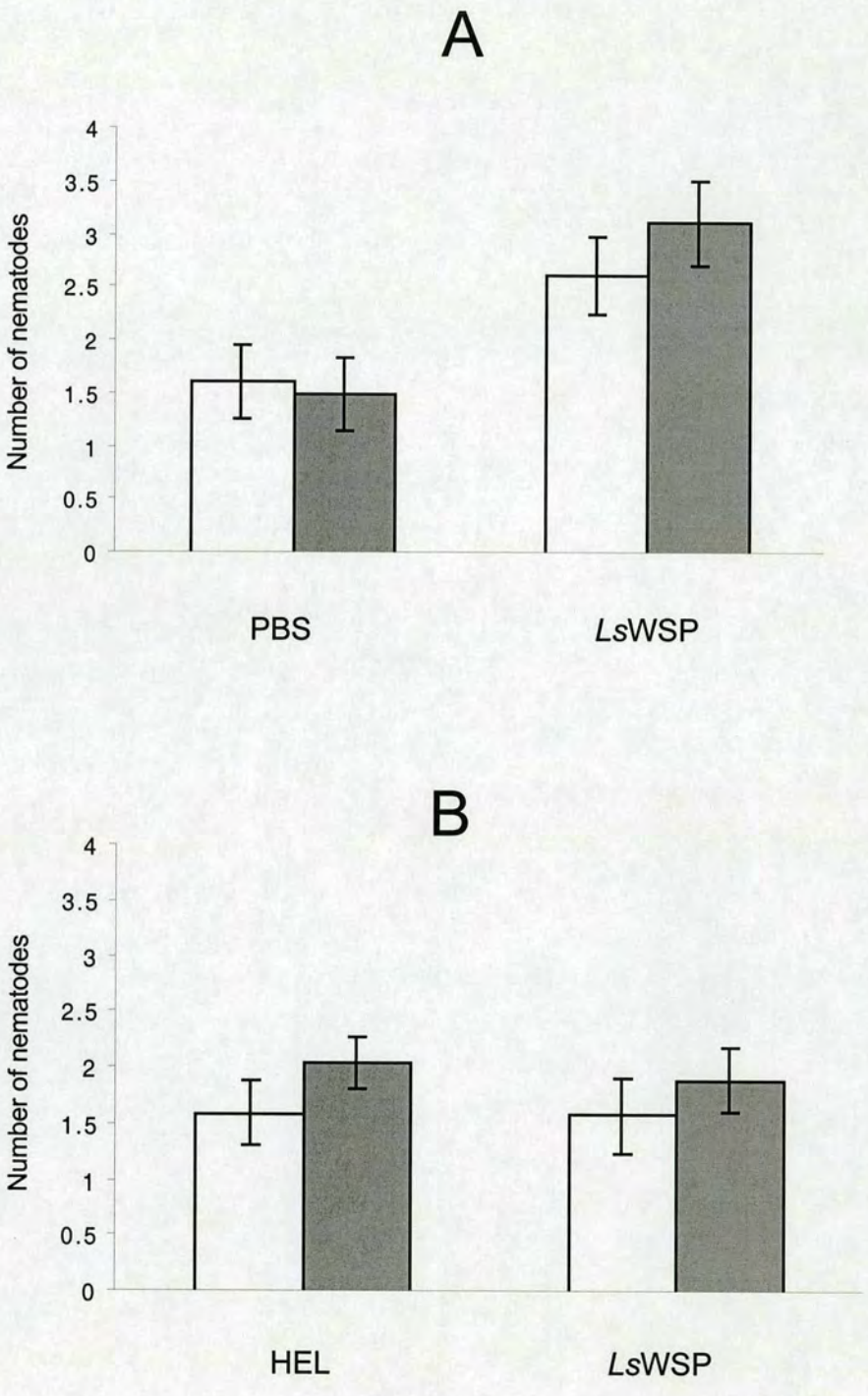


Figure 8:3 The average number of *L. sigmodontis* males and females surviving to day 6O PI in vaccinated and control animals. Each graph represents data pooled from two experiments. A shows the parasites recovered from animals vaccinated with *Ls*WSP in Freund's adjuvant or PBS in Freund's adjuvant. B shows parasites recovered from animals vaccinated with *Ls*WSP in alum or hen egg lysozyme (HEL)in alum. The number of males are represented by the white bars and the number of females are represented by the grey bars. The error bars represent the SEM of the pooled data for each group.

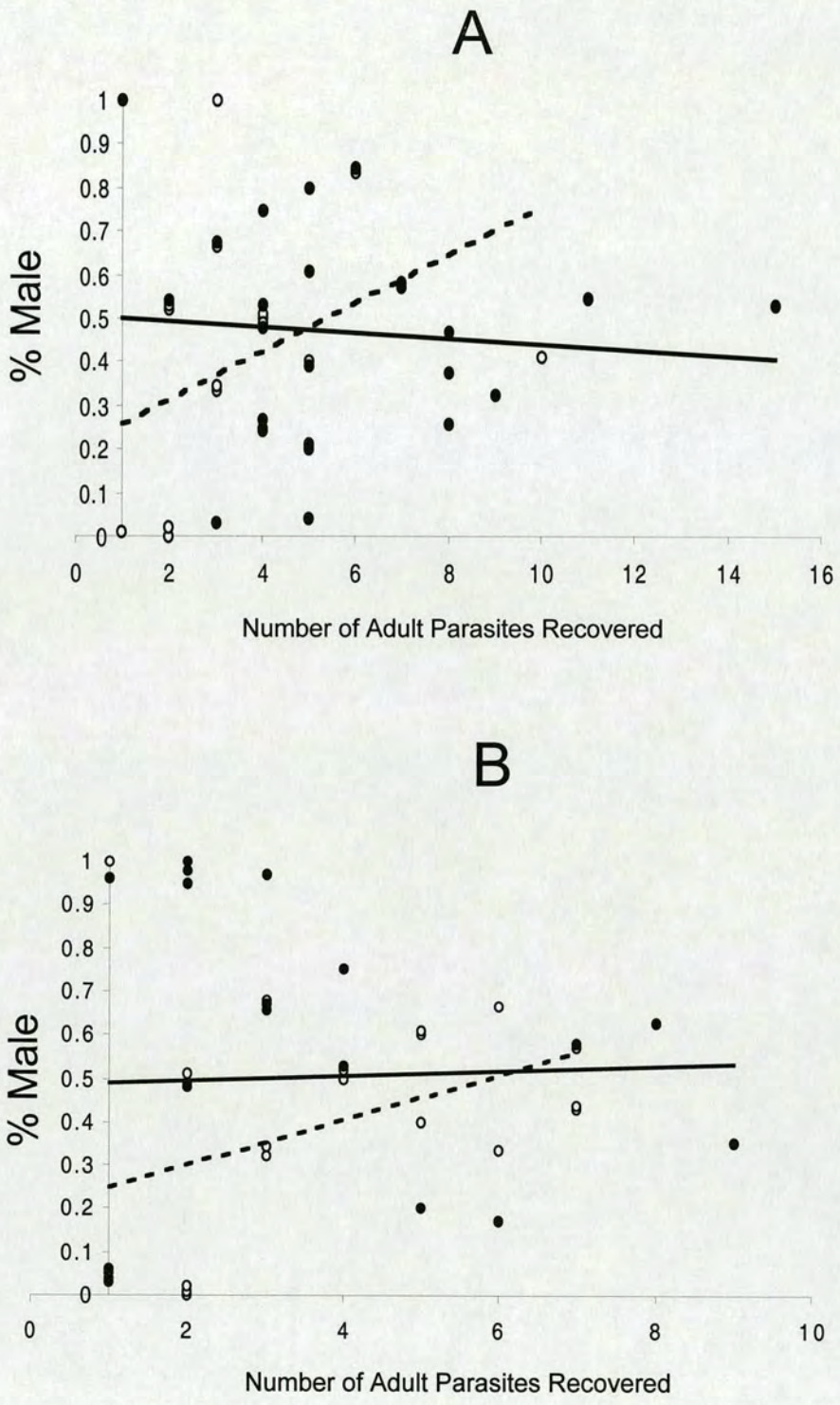


Figure 8:4. The sex ratio of *L. sigmodontis* adult parasites recovered from vaccinated mice at 60 days post-infection. Pooled data from two experiments for each adjuvant is shown. Freund's adjuvant was used in A and alum was used in B. Control animals are represented by the white circles and vaccinated animals are represented by the black circles. The dashed line in each case represents the least-square best fit line for control animals. The solid line represents the least-square best fit line for animals vaccinated with *Ls*WSP.

in alum (Fig. 8:4B) there was no bias in the sex of parasites that were able to survive regardless of the total number of parasites recovered.

When 5 or more parasites were recovered we did not observe a parasite sex bias for either of the adjuvants used in these experiments (Fig. 8:4) so we examined the number of animals that had female biased parasite recovery when the recovery rate was less than or equal to 4 adult nematodes. We tested for differences in the number of animals that did, and did not, have female biased parasite recovery between animals vaccinated with *Ls*WSP, and sham vaccinated animals, but we did not find any statistical differences for either adjuvant used (Logistic regression $P>0.05$ in both cases).

8:3 Discussion

The role of intracellular *Wolbachia* in the biology of filarial nematodes is presently unclear (Taylor and Hoerauf 1999). Studies of a variety of filarial infections, in hosts treated with antibiotics, indicate that *Wolbachia* may be directly involved in moulting of larvae (Bosshardt *et al.*, 1993; Casiraghi *et al.*, 2003), fertility of adult filarial nematodes (Bandi *et al.*, 1999; Hoerauf *et al.*, 1999; Townson *et al.*, 2000) and even adult survival (Langworthy *et al.*, 2000; Townson *et al.*, 2000). Nevertheless these effects could be caused by the mass release of toxic products from dead and dying bacteria rather than the absence of *Wolbachia per se*. Indeed recent studies indicate

that tetracycline treatment does not completely clear filarial nematodes of *Wolbachia* bacteria (Casiraghi *et al.*, 2003).

We hypothesised that, in addition to having direct effects on their filarial nematodes hosts, it was possible that the anti-*Wolbachia* immune responses observed in filarial nematode infection (Lamb *et al.*, 2004; Simon *et al.*, 2003; Bazzocchi *et al.*, 2000a; Punkosdy *et al.*, 2001) may indirectly affect the biology of filarial nematodes. Previous studies indicate that immune responses elicited by *Wolbachia* may be required for granuloma formation around adult filarial nematodes (Brattig *et al.*, 2001) and that the LPS-like molecules contained on *Wolbachia* can induce inflammatory responses (Taylor *et al.*, 2000) that somehow damage the fertility of adult nematodes (Pfarr *et al.*, 2003). This evidence suggests that *Wolbachia* can act indirectly through the mammalian immune system to impair the survival and transmission of their filarial nematode host.

Most L3 stages infect hosts in endemic areas that already have immune responses to WSP (Lamb *et al.*, 2004). In this study we have examined the effect of immune responses to WSP on incoming filarial infection. We have found that under conditions whereby the anti-WSP immune response contains both type 1 and type 2 components (Fig. 8:1B), the anti-*Wolbachia* response can promote the establishment of filarial nematode infection (Fig. 8:2A). This effect is correlated with the presence of type 1 immune components because we observed no enhancement in the establishment of adult *L. sigmodontis* when anti-*Wolbachia* immune responses were predominately

type 2 (Figs. 8:1B and 8:2B). Although it is difficult to explain how immune responses generated against a target that is contained within nematode tissues (*Wolbachia*) could evolve to promote the establishment of incoming filarial nematodes, this data is consistent with hypothesis that type 1 immune responses are needed for filarial parasite development (data reviewed by Ravindran 2001).

Females contain a higher number of *Wolbachia* in their tissues than males (Taylor *et al.*, 1999). Therefore it is likely that female filarial nematodes may have a greater potential to initiate anti-*Ls*WSP responses, rather than the males. If anti-*Wolbachia* immune responses harm filarial nematodes (Brattig *et al.*, 2001; Pfarr *et al.*, 2003) this may reduce the number of competing females that are able to establish. However if anti-WSP immune responses help individual nematodes to establish, as indicated in these experiments (Fig. 8:2A), artificial creation of these immune responses may allow males to establish as well as females. We did not observe any decrease in the average number of females that were able to survive in these experiments (Fig. 8:3). Rather there was an overall increased number of adult parasites that were able to establish in mice vaccinated with *Ls*WSP in Freund's adjuvant. This difference arose because of trends towards increased numbers of both male and female nematodes that were able to establish infection in this group of mice (Fig. 8:3A).

Interestingly, if females are responsible for anti-WSP responses, and these responses preferentially increase male survival, this may be evidence that *Wolbachia* initiate immune responses to facilitate filarial nematode mating, and thus their own

transmission. However we did not observe any increase in the proportion of animals that became microfilaraemic in these experiments (data not shown) although quantification of the number of circulating Mf in microfilaraemic mice may clarify whether anti- *Ls*WSP responses increased the fecundity of the established adult female nematodes.

There is currently no evidence to suggest that the L3 innoculum used to set up infections with *L. sigmodontis* is sex-biased. Nevertheless we observed that when conditions for establishment of *L. sigmodontis* infection are unfavourable, females are more likely than males to establish (Fig. 8:4). This data is not unique as this phenomenon has previously been demonstrated in a range of several different nematode infections (Kwong and Dobson 1982; Tingley and Anderson 1986; Stein *et al.*, 1996). Additionally a comparison of different populations of experimental nematode infections by Poulin (1997) demonstrated that populations that inherently reach higher densities are less female biased. Interestingly the apparent density dependence of the sex ratio of adult parasites recovered at 60 days post-infection with *L. sigmodontis* in the control animals in these experiments was not observed in the groups of animals with pre-existing *Ls*WSP responses (Fig. 8:4). This happened independently of whether there was an overall increase in parasite recovery rate or the adjuvant used to initiate the anti- *Ls*WSP response (Fig. 8:4). It is unclear whether this effect was due to a reduction in female establishment in unfavourable conditions when there are pre-existing anti-*Ls*WSP immune responses, or whether male filarial nematodes became more robust.

We have measured antibody responses against *Ls*WSP as a measure of the immune response against this protein. However because *Wolbachia*, and therefore WSP, are contained within the tissues of filarial nematodes, it is likely that other components of this artificially created immune response facilitate the effects on filarial nematode biology we have observed. Further characterisation of anti-WSP responses, and under what conditions they naturally occur in filarial nematode infection, are needed to interpret the indirect effects of *Wolbachia* exert on filarial nematode biology via the mammalian immune system.

CHAPTER 9

Summary and General Discussion

9:1 Evaluation of the *Litomosoides sigmodontis* model as a model for the establishment of filarial infection.

The majority of work in this thesis has made use of the murine filarial model *Litomosoides sigmodontis*. We have investigated several aspects of filarial nematode biology by studying the establishment of infection in susceptible BALB/c and resistant C57BL/6 mice (Petit *et al.*, 1992). By using *L. sigmodontis* infection as a model for filarial infection, we have been able to study filarial nematode biology in the context of the development of filarial nematodes from L3 stages to patent adults in the mammalian host.

Whilst this work would not have been possible using any other model, the data for filarial nematode establishment obtained from the experiments in this thesis has been variable, reducing the ability to detect differences in the parameters measured. We were able to overcome this to a certain extent by infecting larger numbers of animals in some experiments (Chapter 5 and Chapter 8). However even with larger groups of animals, differences between treatment groups could only be detected using meta-analysis. The variability in the outcome of *L. sigmodontis* infection was particularly a problem for analysis of immune responses in co-infection studies with *L. major*

(Chapter 6). Larger groups could not easily be used in these studies to assess immune responses using ELISPOT.

It is currently unknown why the establishment of *L. sigmodontis* in inbred mouse strains varies to such a large extent. However variation in adult nematode recovery appears to be present regardless of the method used to introduce *L. sigmodontis* infective stages into experimental mice (Martin *et al.*, 2001; Volkmann *et al.*, 2001; Le Goff *et al.*, 2002). Additionally only about half of BALB/c mice appear to develop circulating microfilaria (Mf) (Petit *et al.*, 1992; Le Goff *et al.*, 2002). The *L. sigmodontis* model is an invaluable tool to examine the development, and establishment of primary filarial infection in the mammalian host. Nevertheless, on a practical level, work aimed at understanding the basis of variation in the establishment of this infection within groups of inbred mice would be useful in the interpretation of experimental results. Such studies in themselves, may also be a better model to use to understand resistance and susceptibility to filarial infection than comparisons of strains of mice that are overall resistant or susceptible. As demonstrated data concerning the role of IL4 in the establishment of filarial infection (Chapter 3, Volkmann *et al.*, 2003), the basis of strain resistance and susceptibility to *L. sigmodontis* infection can be due to different immunological factors (Volkmann *et al.*, 2003; Le Goff *et al.*, 2002; Le Goff *et al.*, 2000b; Martin *et al.*, 2000a).

9:2 The type 2 response elicited by filarial nematodes.

We have shown that IL4 is a necessary immunological component that facilitates the resistance of C57BL/6 mice to the establishment of *L. sigmodontis* infection (Chapter 3; Le Goff *et al.*, 2002). Further studies indicated that both the innate and acquired immune arms of the immune response must produce IL4 in order to provide protection (Chapter 4). We have hypothesised that the IL4 evoked from the innate immune response is necessary to instruct the acquired immune response, rather than directly attacking incoming parasites. Several pieces of evidence indicate that this hypothesis is correct. C57BL/6 IL4-/- mice have a similar recovery rate to wild type C57BL/6 mice (Fig. 3:1A) indicating that the IL4 produced in the early defence against establishment of *L. sigmodontis* infection plays little role in protection in this mouse strain. Additionally chimeric C57BL/6 mice that can only produce IL4 from the one arm of the immune response are as susceptible to the establishment of *L. sigmodontis* infection as C57BL/6 IL4-/- mice (Figs. 4:6A and 4:7).

IL4 cannot be described as a general resistance factor to the establishment of *L. sigmodontis* infection. BALB/c mice produce IL4 in response to *L. sigmodontis* infection and yet are a susceptible strain (Fig. 3:2B). This suggests that in BALB/c there is a susceptibility factor that is dominant over IL4, and absent in C57BL/6 mice. Examination on the immune response generated by filarial infection in BALB/c mice indicates that one such possible factor could be IL5. Alongside IL4, this cytokine also predominates in murine filarial infection and has been shown to contribute to the

observed formation of neutrophilic granulomas in this mouse strain around 80 days post- infection (Al-Qaoud *et al.*, 2000). This cytokine provides some protection against *L. sigmodontis* in BALB/c mice (Volkmann *et al.*, 2003; Martin *et al.*, 2000a) but is not alone a protective factor in C57BL/6 mice (Le Goff *et al.*, 2000b).

Alternatively components of the type 1 response may be protective. IFNγ has been found to be protective in BALB/c mice once an infection has established (Volkmann *et al.*, 2003). However studies investigating the role of type-1 associated immune responses in resistance to the establishment of *L. sigmodontis* infection in C57BL/6 mice have not yet been published. Future studies in this area will be interesting because the ability of C57BL/6 mice to mount a type 1 response, and the speed that they do so, may explain why they are resistant. The resistance and susceptibility pattern observed in BALB/c and C57BL/6 mouse strains against the establishment of *L. sigmodontis* infection is similar to that observed against the protozoan parasite *L. major* (Sacks and Noben-Trauth 2002). *L. major* initially evokes a type 2 response and C57BL/6 mice are resistant because they are able to subsequently mount a type 1 response. On the other hand BALB/c mice are unable to mount a type 1 response within the time scale required to contain infection and they succumb to a systemic infection.

Further evidence that a type 1 response may be involved in the resistance of mice to infection with *L. sigmodontis* can be found in co-infection studies with type 1 inducing organisms. We have shown that mice co-infected with either the rodent malaria

parasite *P. chabaudi*, or *L. major*, can result in an acceleration of adult nematode death (Figs. 5:4B, 5:4D and 6:6). However we were not able to detect an increased anti-filarial type 1 response in either of these co-infection studies (Chapters 5 and 6). Nevertheless there was a decrease in the amount of IL4 elicited by *L. sigmodontis*, although this could be the result, rather than a cause, of the decreased number of live parasites.

Although it is possible that type 1 responses are protective in mice, the evidence presented above largely concerns the immune attack of established parasites. It has been suggested that type 1 responses can assist incoming juvenile parasites to develop and establish (Ravindran 2001). Consistent with this hypothesis, the presence of type 1 anti-WSP responses enhanced the recovery of adult *L. sigmodontis* parasites at day 60 post-infection (Fig. 8:2A). Therefore, type 1 responses may perform different roles against different stages of filarial nematodes.

9:3 Endosymbiotic bacteria: Filarial nematodes are two organisms in one package.

Work in this thesis has examined the effects of the filarial intracellular bacterium *Wolbachia* on the biology of filarial nematodes. We have determined that *Wolbachia* are seen by the immune system in an area where filarial nematode infection is endemic, and antibody responses against the surface protein of *Wolbachia* (WSP) are generated largely by the L3 stage (Chapter 7). Subsequently we have also shown that

pre-existing type 1 immune responses to WSP can increase the establishment of adult filarial nematodes and alter the sex ratio of filarial nematodes that are able to establish in primary infection (Chapter 8).

Currently little is known about the role of *Wolbachia* in filarial nematode biology. These bacteria have been coined as endosymbionts of their filarial nematode host, largely based on experiments that have removed the bacterium from the nematode using the antibiotic tetracycline. Effects of this treatment on filarial nematodes, including infertility and the inability of nematode to develop beyond the L3 stage, have been attributed to the loss of *Wolbachia*. However these effects could also be due to toxic products released from dead and dying bacteria.

We have investigated the relationship of *Wolbachia* with their filarial nematode host by asking whether these bacteria play any role in the biology of filarial nematodes via the mammalian immune system. After establishing that anti-WSP responses occur in primary filarial infection (Chapter 7), we have observed that pre-existing anti-WSP immune responses can enhance the establishment of primary filarial infection (Chapter 8). This indicates that anti-WSP immune responses can alter the dynamics of filarial nematode infection in a positive way.

The mechanisms by which anti-WSP responses may have effects on filarial nematode establishment were not examined in this thesis. We have measured antibody responses to WSP as a measurement of anti-WSP immune responses (Fig. 8:1). Nevertheless it is

difficult to imagine how antibody responses could target *Wolbachia* as they are intracellular bacteria that live within the tissues of filarial nematodes. It is our hypothesis that other components of the anti-*Wolbachia* immune response could facilitate increased filarial nematode establishment, perhaps by altering the immune response generated against the nematode host. We measured no differences in IL4 or IFNγ secretion targeted against adult filarial nematodes from splenocytes of mice that had pre-existing anti-*Wolbachia* immune responses compared with control mice (data not shown). However there are many other aspects of anti-filarial immune responses that could be altered by anti-*Wolbachia* immune responses and facilitate increased nematode establishment. IL10 and TGFβ are two cytokines that have been linked with suppression of some anti-filarial immune responses in mice (Osborne and Devaney 1999) and humans (King *et al.*, 1993; Mahanty and Nutman 1995). Investigation of the induction of these cytokines by *Wolbachia*, especially in the vaccination studies in this thesis, may shed further light on the effects of pre-existing anti-WSP responses on filarial nematode establishment.

Although we have observed effects of pre-existing anti-WSP responses on filarial nematode establishment, there are several factors that make the interpretation of these results difficult. Firstly we have only vaccinated with WSP, and not whole *Wolbachia*. A study by Jiggins *et al.* (2002) found no evidence of strong selection pressure on WSP from filarial nematodes, as might be expected if this protein evoked mammalian immune responses that enhance filarial nematode establishment. Secondly it is unknown whether anti-*Wolbachia* immune responses can be generated by live filarial

nematodes. It would be possible to test this by examining medium from *in vitro* cultures of different stages of filarial nematodes, and from cultures of moulting filarial nematodes as it is possible that *Wolbachia* may be shed during the moulting. It is difficult to envisage how anti-*Wolbachia* immune responses could evolve to enhance the establishment of their filarial nematode host unless these responses are generated from live nematodes.

9:4 Conclusion

In conclusion, the work in this thesis has validated the use of the *L. sigmodontis* model in studies of filarial nematode establishment. By examining anti-filarial immune responses in mice resistant to the establishment of *L. sigmodontis*, we have made some progress in understanding components of the immune responses that are essential for host resistance. We have determined that immune responses generated against co-infecting organisms are immunological factors that can alter resistance and susceptibility to *L. sigmodontis* infection. Finally we have been able to investigate the relationship between *L. sigmodontis* and the filarial intracellular bacterium *Wolbachia* and found that these immune responses may, in some circumstances, increase the susceptibility of a host to the establishment of infection with filarial nematodes.

APPENDIX

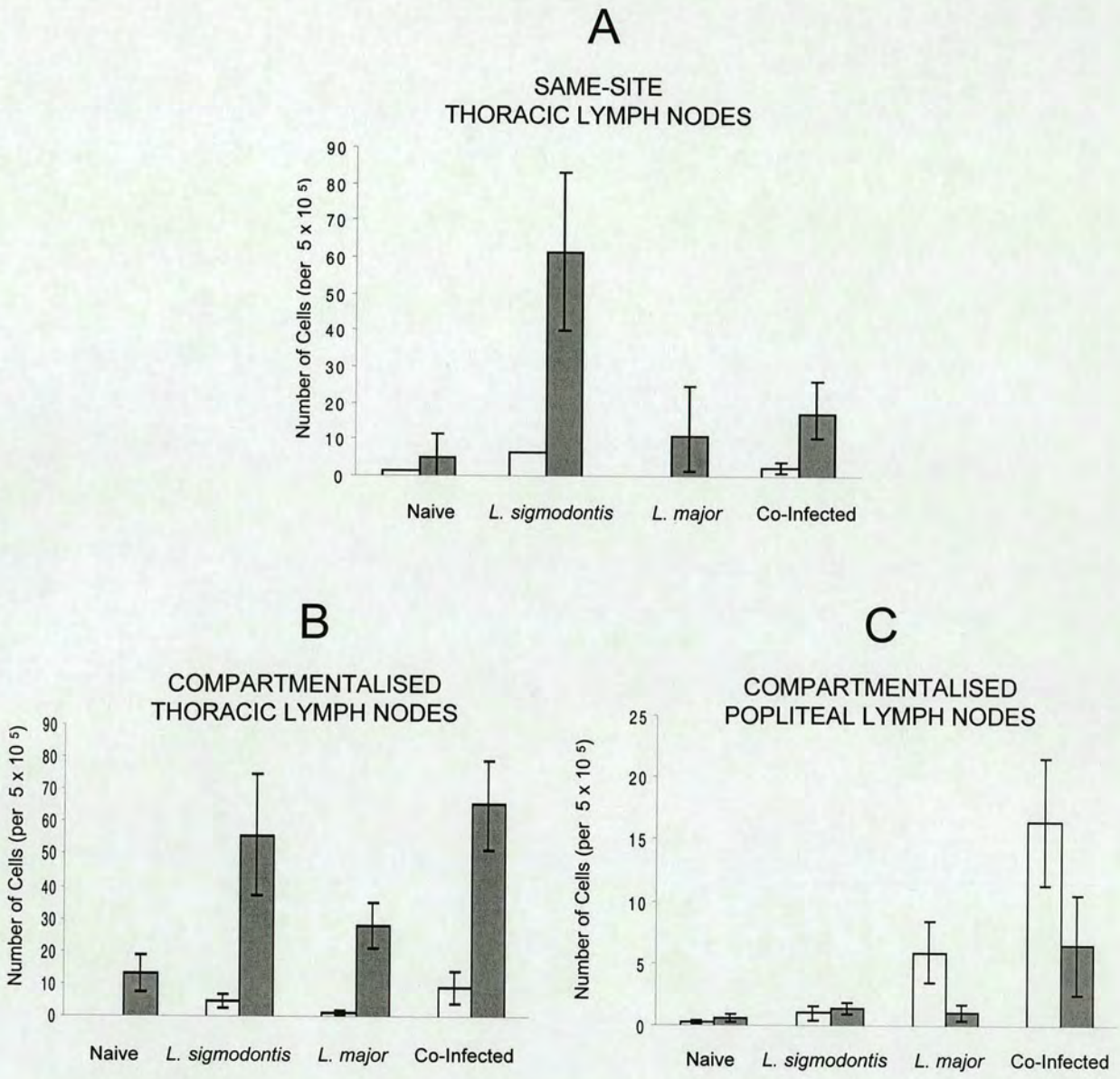


Figure Ap1. *Ex vivo* immune responses in mice singly and co-infected with *L. major* and *L. sigmodontis* in the lymph nodes of compartmentalised and same-site co- infection at 40 days post - *L. major* infection. The response in the thoracic lymph nodes of same-site co-infection is shown in A. This data is compared with the response in the thoracic lymph nodes (B) and the popliteal lymph nodes (C) in compartmentalised co-infection. The number of cells secreting IFNγ are represented by the white bars and the number of cells secreting IL4 are represented by grey bars. 3 x 10^5 cells were assayed although these results were multiplied to graphically represent 5 x 10^5 lymph node cells. The error bars represent the maximum and minimum values in A and the SEM of data pooled from two experiments in B and C. The response of naive animals are shown for comparison but were not included in any of the analyses.

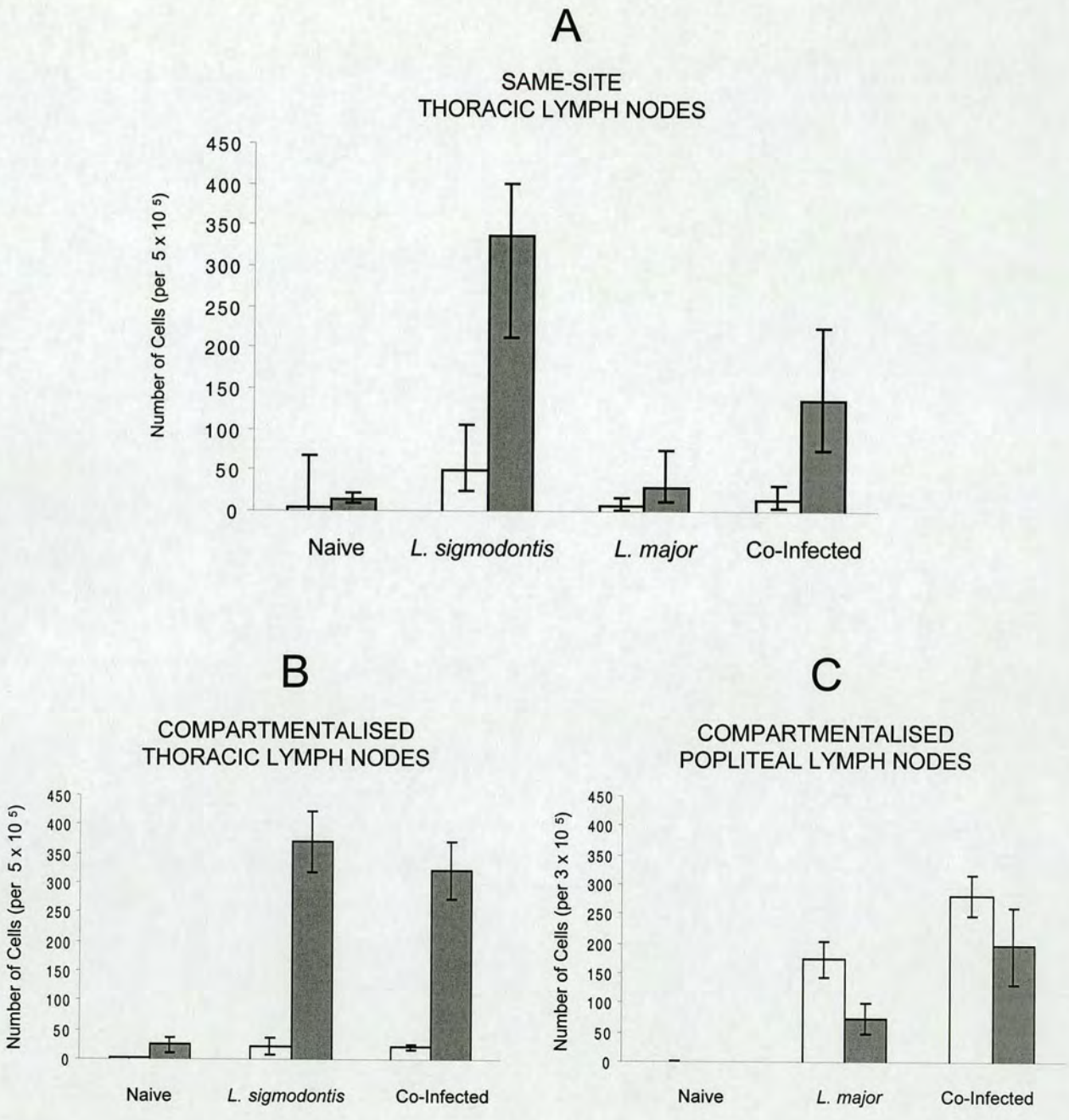


Figure Ap2. Immune responses to the lymphocyte mitogen Con A in the thoracic lymph nodes of singly-infected and co-infected mice in same-site co-infection at 40 days post-infection with *L. major* (A).The number of cells secreting IFNγ are represented by the white bars and the number of cells secreting IL4 are shown by the grey bars. 3 x 10^5 thoracic lymph node cells were analysed although these results were multiplied to graphically represent analysis with 5 x 10^5 thoracic lymph node cells. In A the error bars represent the maximum and minimum values for each group of 5 mice. In B the error bars represent the SEM and are represent pooled values obtained from 2 different experiments. The responses of naive animals are shown for comparison but were not included in any of the analyses.

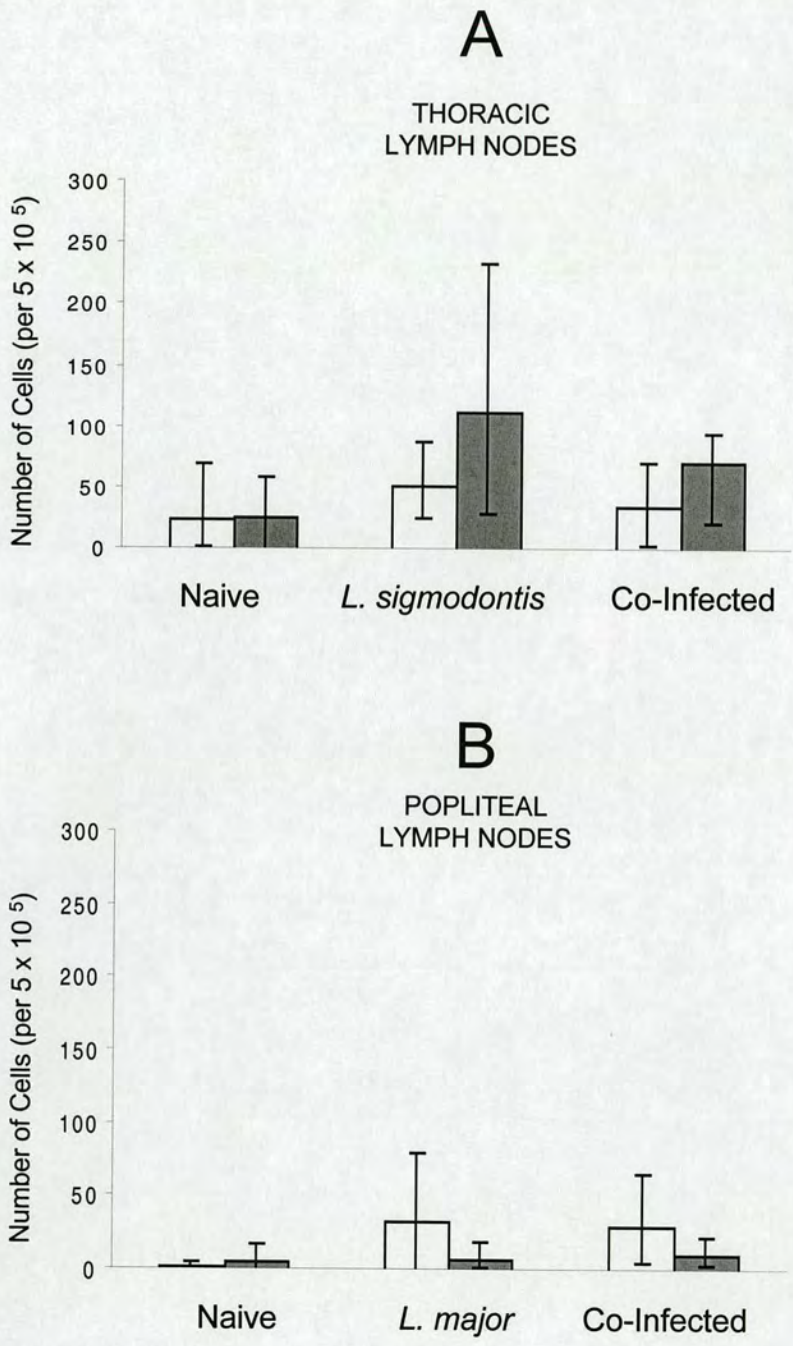


Figure Ap3. Immune responses to *L. major* and *L. sigmodontis* in compartmentalised co-infection at day 14 post-infection with *L. major*. The number of cells secreting IFNγ (white bars) and IL4 (grey bars) against *L. sigmodontis* in the thoracic lymph nodes (A) and against *L. major* in the popliteal lymph nodes (B) are shown. 3 x 10^5 thoracic or popliteal lymph node cells were assayed although these results were multiplied to graphically represent 5 x 10^5 lymph node cells. The error bars represent the maximum and minimum values for each group and cytokine. The responses of naive animals are shown for comparison but were not included in any of the analyses.

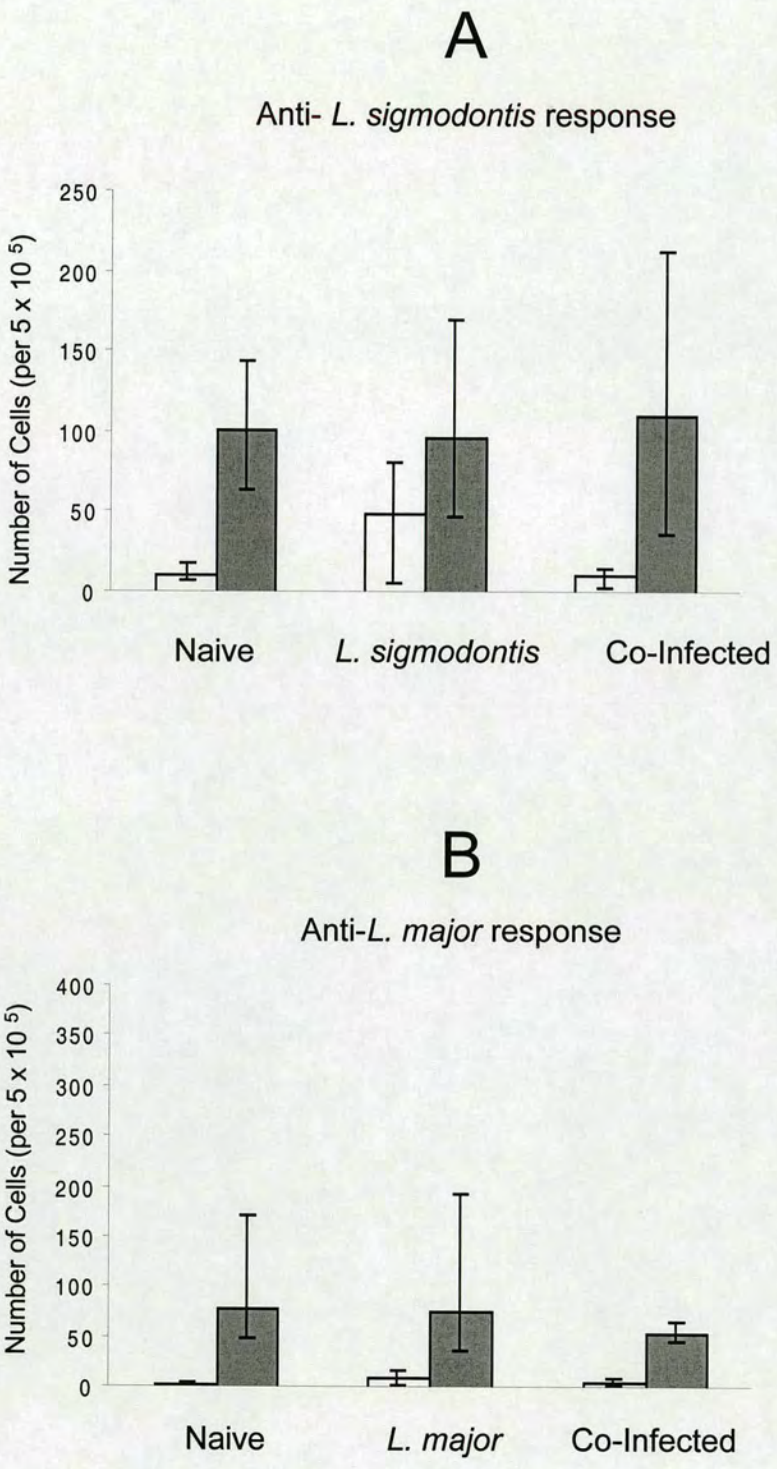


Figure Ap4. Splenic immune responses to *L. major* and *L. sigmodontis* in compartmentalised co-infection at day 14 post-infection with *L. major.* The number of cells secreting IFNγ (white bars) and IL4 (grey bars) against *L. sigmodontis* (A) and *L. major* (B) are shown. The error bars represent the maximum and minimum values for each group of 5 mice. The responses of naive animals are shown for comparison but were not included in any of the analyses.

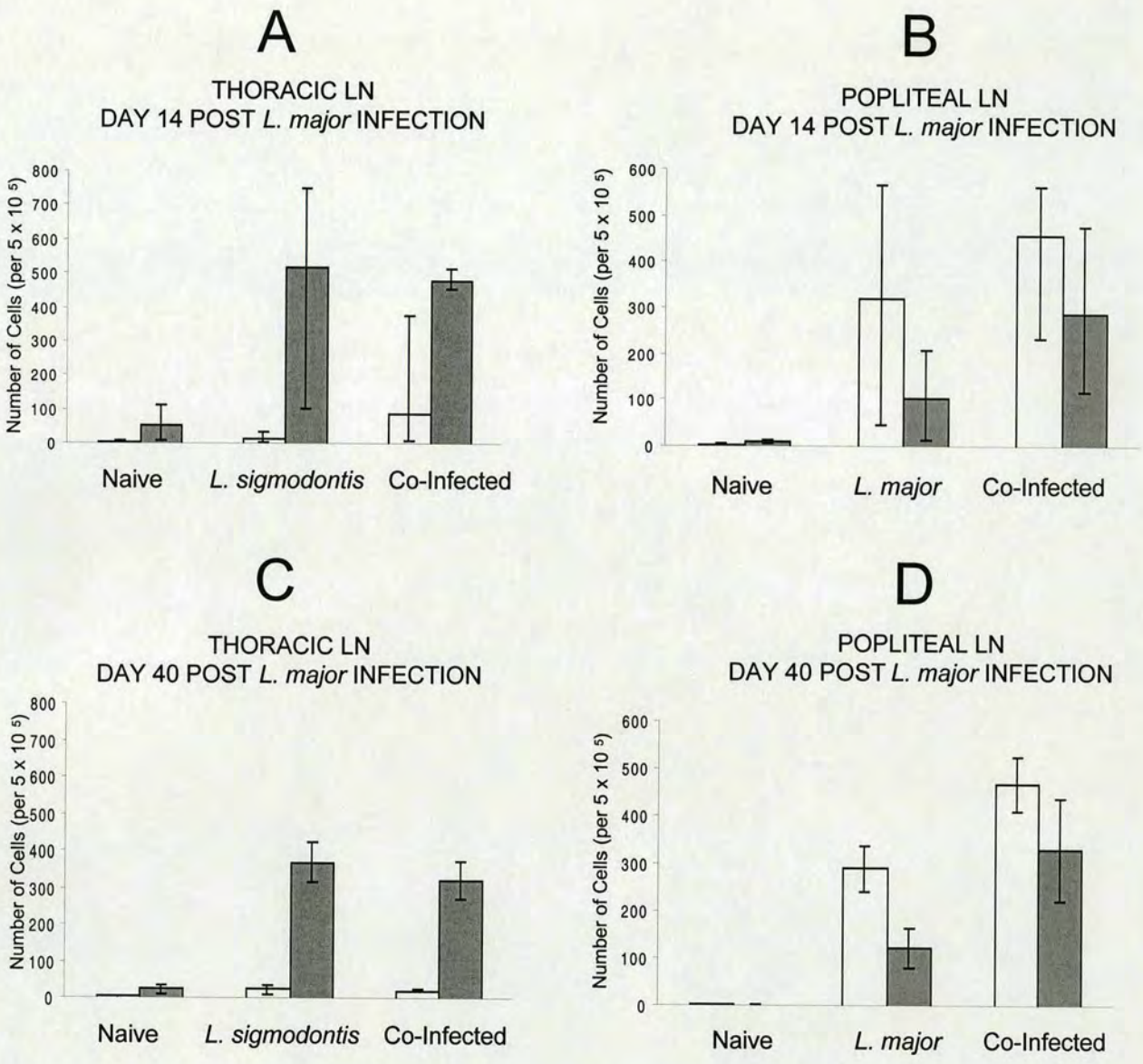


Figure Ap5. Immune responses to the lymphocyte mitogen Con A in the thoracic lymph nodes (A and C) and in the popliteal lymph nodes (B and D) of co-infected mice and singly infected mice in compartmentalised co-infection. The responses of mice 14 days post-*L. major* infection (A and B) and 40 days post -*L. major* infection (C and D - duplicated from Fig. Ap2 for comparison) are shown. The number of cells secreting IFNγ are represented by the white bars and the number of cells secreting IL4 are shown by the grey bars. 3 x 10^5 thoracic and popliteal lymph node cells were assayed although these results were multiplied to graphically represent 5 x 10^5 lymph node cells. In A and B the error bars represent the maximum and minimum values for each group of 5 mice. Figures C and D are the pooled values obtained from 2 different experiments and the error bars represent the SEM. The responses of naive animals are shown for comparison but were not included in any of the analyses. LN - lymph nodes.

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